IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

HUMAN GENOME SCIENCES, INC.,	)	
	)	Civil Action No. _____
Plaintiff,	)	
	)	
v.	)	
	)	
AMGEN INC. and IMMUNEX CORP.,	)	
	)	
Defendants.	)	

COMPLAINT

NATURE OF ACTION

1. This is an action under 35 U.S.C. §146 to review the judgment entered July 27, 2007 by the Board of Patent Appeals and Interferences ("the Board") of the United States Patent and Trademark Office ("PTO") in Interference 105,381 ("the '381 Interference").

JURISDICTION AND VENUE

2. This Court has subject matter jurisdiction of this action pursuant to 35 U.S.C. § 146 and 28 U.S.C. §§1331 and 1338.

3. Venue is proper in this judicial district pursuant to 28 U.S.C. § 1391(c).

PARTIES

4. Human Genome Sciences, Inc. ("HGS") is a corporation organized and existing under the laws of the State of Delaware and has a place of business in Rockville, Maryland.

5. Upon information and belief, Defendant Amgen Inc. ("Amgen") is a corporation organized and existing under the laws of the State of Delaware and has a place of business at One Amgen Center Drive, Thousand Oaks, California.

6. Upon information and belief, Defendant Immunex Corp. ("Immunex") is a wholly-owned subsidiary of Amgen organized and existing under the laws of the State of Washington and has a place of business in Thousand Oaks, California.

7. Upon information and belief, Defendant Immunex is registered to do business in Delaware.

8. Upon information and belief, Defendants derive substantial revenue from products that are used or consumed in Delaware.

THE PATENT INTERFERENCE

9. This action arises from the judgment entered on July 27, 2007 by the Board in the '381 Interference. A true and correct copy of the judgment, entitled "Judgment – Order to Show Cause," Paper 135, is attached as Exhibit A.

10. The '381 Interference involved patent claims relating to a purified TRAIL-R polypeptide molecule.

11. HGS is the owner by assignment of the entire right, title and interest in and to the invention disclosed in U.S. Patent Application Serial No. 10/005,842 filed December 7, 2001 ("the '842 application"), entitled "Death Domain Containing Receptor 5," in the names of Jian Ni, Reiner L. Gentz, Guo-Liang Yu, and Craig A. Rosen (collectively "Ni"). A copy of the '842 application (without the preliminary amendment) is attached as Exhibit B.

12. Upon information and belief, Defendants are the owner of the entire right, title and interest in and to U.S. Patent No. 6,642,358, granted November 4, 2003 ("the '358 patent") based on Application Serial No. 09/578,392 filed May 25, 2000, entitled "Receptor That Binds TRAIL" in the names of Charles Rauch and Henning Walczak (collectively "Rauch"). Immunex is the recorded assignee of the '358 patent. Upon information and belief, both Amgen and Immunex have actively participated in the prosecution of the '358 patent and the '381 Interference. A copy of the '358 patent is attached as Exhibit C.

13. The Board declared and instituted the '381 Interference.

14. On July 27, 2007, the Board issued the Judgment – Order to Show Cause in the '381 Interference adverse to HGS and Ni, and favorable to Immunex and Rauch, wherein, with its March 26, 2007 Decision – Motions (Paper 101), its May 30, 2007 Decision-Rehearing (Paper 113), and its July 27, 2007 Decision - Order to Show Cause (Paper 134) the Board, and its other decisions, erroneously ruled, contrary to fact and law, *inter alia*, that:

a. Ni was not entitled to substitute Count 1 of the interference with a proposed Count 2, denying Ni Substantive Motion 1;

b. Ni's entitlement to a priority date for proposed Count 2 was moot, dismissing Ni Substantive Motion 2;

c. as to Count 1, Ni was not entitled to the benefit of priority of the March 17, 1997 filing date of provisional application 60/040,846, denying Ni's Substantive Motion 2, in part;

d. all Rauch's involved claims are patentable as not anticipated under 35 U.S.C. § 102(e) by U.S. Patent No. 6,872,568, denying Ni Substantive Motion 3;

e. denying Ni Miscellaneous Motion 4 to exclude certain exhibits from evidence;

f. as to Count 1, Rauch was entitled to the benefit of priority of the March 28, 1997 and June 4, 1997 filing dates of applications 08/829,536 and 08/869,852, respectively, granting Rauch Substantive Motion 1, in part;

g. Ni's involved claims are unpatentable under 35 U.S.C. § 102(e) as anticipated by U.S. Patent No. 6,072,047, granting Rauch Substantive Motion 3, in part; and

h. the Board need not reach the issue of whether of Rauch's involved claims were invalid as anticipated under 35 U.S.C. § 102(g) based on Ni's proof of prior invention of the claimed subject matter.

15. HGS is dissatisfied with the Board's decisions and judgment.

16. HGS is dissatisfied with and seeks review and reversal (or in the alternative vacation and remand) of every decision in the '381 Interference in which the Board ruled against Ni, denied or dismissed the relief sought by Ni, or granted the relief sought by Rauch.

17. HGS has not sought review of the Board's Decisions and Final Judgment by the United States Court of Appeals for the Federal Circuit, and pursuant to 35 U.S.C. §

146, has elected to file suit in this Court for dissatisfaction with the Board's Decisions and Final Judgment.

18. The Board's judgment in the '381 Interference was erroneous, and HGS is entitled to judgment in this action correcting the erroneous judgment and rulings of the Board, based on the record before the Board and any additional evidence that HGS may introduce in this action.

19. Ni has priority as to the subject matter at issue in the '381 Interference and Rauch's involved claims are unpatentable, *inter alia*, under 35 U.S.C. § 102(g).

20. Ni's involved claims are patentable.

21. Rauch's involved claims are unpatentable.

WHEREFORE, HGS demands judgment that:

a. The June 27, 2007 decision in Interference 105,381 be vacated;

b. The Board's judgment be reversed;

c. Ni's involved claims are patentable;

d. Rauch's involved claims are unpatentable;

e. all Rauch's involved claims are unpatentable as anticipated under 35 U.S.C. § 102(e) by U.S. Patent No. 6,872,568;

f. Rauch is not entitled to the benefit of priority of the March 28, 1997 and June 4, 1997 filing dates of applications 08/829,536 and 08/869,852, respectively;

g. Ni's involved claims are patentable under 35 U.S.C. § 102(e) as not anticipated by U.S. Patent No. 6,072,047;

h. Ni is entitled to the benefit of priority of the March 17, 1997 filing date of provisional application 60/040,846;

i. Ni has priority as to the subject matter at issue in the '381 Interference under 35 U.S.C. § 102(g), or in the alternative, that the case be remanded to the Board for a determination of priority;

j. Rauch's involved claims are unpatentable under 35 U.S.C. § 102(g), or in the alternative, that the case be remanded to the Board for a determination of priority;

k. Ni is entitled to substitute Count 1 of the interference with a proposed Count 2;

l. Ni has priority as to the subject matter of proposed Count 2;

m. Ni has priority as to the subject matter of proposed Count 1;

n. That every decision in the interference in which the Board ruled against Ni be reversed or in the alternative vacated and remanded;

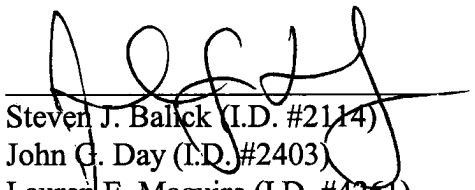
o. That every decision in the interference in which the Board denied or dismissed the relief sought by Ni be reversed or in the alternative vacated and remanded;

p. That every decision in the interference in which the Board granted the relief sought by Rauch be reversed or in the alternative vacated and remanded;

q. Costs and attorneys fees be awarded in favor of HGS against Immunex; and

r. Such other and further relief as may be appropriate.

ASHBY & GEDDES



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Dated: August 30, 2007
183700.1

EXHIBIT A

The opinion in support of the decision being entered today is not binding precedent of the Board

Paper 135
Filed: 27 July 2007

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Human Genome Sciences, Inc.,
Junior Party
(Application 10/005,842-IFW
Inventors: Jian Ni, Reiner L. Gentz, Guo-Liang Yu and Craig A. Rosen),
v.
Immunex Corp.,
Senior Party
(Patent 6,642,358
Inventors: Charles Rauch and Henning Walczak).

Patent Interference No. 105,381 (RES)

Before McKELVEY, *Senior Administrative Patent Judge*, and SCHAFER and MOORE, *Administrative Patent Judges*.

Judgment - Order to Show Cause

Junior Party Human Genome Sciences (HGS) was put under an order to show cause why judgment should not be entered against it. Paper 114.

HGS responded. Paper 115. For the reasons given in the Decision - Order to Show Cause entered concurrently herewith (Paper 134), it is

ORDERED that judgment as to Count 1 (the sole count in the interference; Paper 1, page 6) is awarded against Junior Party HUMAN GENOME SCIENCES;

FURTHER ORDERED that HUMAN GENOME SCIENCES claims 35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-109, 111-116, 127-133, 168-178, 180-203 are unpatentable over priori art 35 U.S.C. § 102(e));

FURTHER ORDERED that Junior Party HUMAN GENOME SCIENCES is not entitled to a patent containing claims 35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-109, 111-116, 127-133, 168-178, 180-203 of Application 10/005,842 (corresponding to Count 1);

FURTHER ORDERED that if there is a settlement agreement, attention is directed to 35 U.S.C. § 135(c);

FURTHER ORDERED that a copy of this JUDGMENT shall be placed in the files of (1) Application 10/005,842 and (2) Patent 6,642,358; and

FURTHER ORDERED that in the event of judicial review, the party seeking judicial review must comply with mandatory notice provisions of 37 C.F.R. § 41.8(b) (2006).

/Fred E. McKelvey/ FRED E. MCKELVEY Senior Administrative Patent Judge	BOARD OF PATENT APPEALS AND INTERFERENCES
/Richard E. Schafer/ RICHARD E. SCHAFER Administrative Patent Judge	
/James T. Moore/ JAMES T. MOORE Administrative Patent Judge	

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1

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EXHIBIT B

Death Domain Containing Receptor 5

Background of the Invention

Field of the Invention

The present invention relates to a novel member of the tumor necrosis factor family of receptors. More specifically, isolated nucleic acid molecules are provided encoding human Death Domain Containing Receptor 5, or simply "DR5." DR5 polypeptides are also provided, as are vectors, host cells, and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR5 activity.

This application claims benefit under 35 U.S.C. § 119(e) to copending U.S. Provisional Application Serial Nos. 60/040,846, filed March 17, 1997 and 60/054,021, filed July 29, 1997, both of which are incorporated herein by reference.

Related Art

Numerous biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intracellular response.

For example, tumor necrosis factors (TNF) alpha and beta are cytokines, which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

Among the ligands, there are included TNF-α, lymphotoxin-α (LT- α, also known as TNF-β), LT-β (found in complex heterotrimer LT-α2-β), FasL, CD40L, CD27L, CD30L, 4-lBBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-lBB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals, 22*:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., *et al., Nature 356*:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C. *et al., Science 259*:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innovation of peripheral structures (Lee, K.F. *et al., Cell 69*:737 (1992)).

TNF and LT-α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT-α, acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT-α are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., *Science 264*:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for transducing signals for programmed cell death (Tartaglia *et al., Cell 74*:845 (1993)).

Apoptosis, or programmed cell death, is a physiologic process essential for the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267:1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267:1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland et *al., Cell* 81:479-482 (1995); A. Fraser, *et al., Cell* 85:781-784 (1996); S. Nagata *et al., Science* 267:1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith *et al., Science 248*:1019-23 (1990); M. Tewari *et al.,* in *Modular Texts in Molecular and Cell Biology* M. Purton, Heldin, Carl, Ed. (Chapman and Hall, London,

1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the *Drosophila* suicide gene, *reaper* (P. Golstein, *et al., Cell 81*:185-186 (1995); K. White *et al., Science* 264:677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan *et al., Cell 81*: 505-12 (1995); M. P. Boldin *et al., J. Biol Chem 270:*7795-8 (1995); F.C. Kischkel *et al., EMBO 14*:5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al., Cell 85:*817-827 (1996); M.P. Boldin *et al., Cell 85:*803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB (L.A. Tartaglia *et al., Immunol Today 13*:151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu *et al., Cell 81*:495-504 (1995); H. Hsu, *et al., Cell 84*:299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation (H. Hsu *et al., Cell 84*:299-308 (1996); H. Hsu, *et al., Immunity 4*:387-396 (1996)).

Recently, a new apoptosis -inducing TNF ligand has been discovered. S.R. Wiley *et al. (Immunity 3*:673-682 (1995)) named the molecule - "TNF-related apoptosis-inducing ligand" or simply "TRAIL." The molecule was also called "Apo-2 ligand" or "Apo-2L." R.M. Pitt *et al., J. Biol. Chem. 271*:12687-12690 (1996). This molecule was also disclosed in co-pending U.S. provisional application no. 60/013,405. For convenience, the molecule will be referred to herein as TRAIL.

Unlike FAS ligand, whose transcripts appear to be largely restricted to stimulated T-cells, significant levels of TRAIL are detected in many human tissues (e.g., spleen, lung, prostate, thymus, ovary, small intestine, colon, peripheral blood lymphocytes, placenta, kidney), and is constitutively transcribed by some cell lines. It has been shown that TRAIL acts independently from the Fas ligand (Wiley *et al., supra*). It has also been shown that TRAIL activates apoptosis rapidly, within a time frame that is similar to death signaling by Fas/Apo-1L, but much faster than TNF-induced apoptosis. S.A. Marsters *et al., Current Biology 6*:750-752 (1996). The

inability of TRAIL to bind TNFR-1, Fas, or the recently identified DR3, suggests that TRAIL may interact with a unique receptor(s).

Several unique receptors for TRAIL have already been identified. In co-pending U.S. provisional patent application no. 60/035,722, DR4, a novel death domain containing receptor for TRAIL, was disclosed. See, Pan *et al., Science 276*,111-113 (April 1997). The TR5 receptor, the subject of co-pending U.S. provisional patent application 60/035,496, has now been shown to bind TRAIL. In co-pending U.S. provisional patent application no. 60/**xxxxxx**, it was predicted that the TR10 receptor would also bind TRAIL, owing to sequence homology with DR4.

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize additional novel receptors that bind TRAIL.

Summary of the Invention

The present invention provides for isolated nucleic acid molecules comprising nucleic acid sequences encoding the amino acid sequence shown in FIG. 1 (SEQ ID NO:2) or the amino acid sequence encoded by the cDNA clone deposited as ATCC Deposit No. 97920 on March 7, 1997.

The present invention also provides recombinant vectors, which include the isolated nucleic acid molecules of the invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of DR5 polypeptides or peptides by recombinant techniques.

The invention further provides an isolated DR5 polypeptide having an amino acid sequence encoded by a polynucleotide described herein.

The present invention also provides diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR5 protein. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR5, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors.

Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional

regulation of several genes. Cellular response to TNF-family ligands include not only normal physiological responses, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis - programmed cell death - is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes. Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers, autoimmune disorders, viral infections, inflammation, graft versus host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

Thus, the invention further provides a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an agonist capable of increasing DR5 mediated signaling. Preferably, DR5 mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an antagonist capable of decreasing DR5 mediated signaling. Preferably, DR5 mediated signaling is decreased to treat a disease wherein increased apoptosis is exhibited.

Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below. Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR5 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By the invention, a cell expressing the DR5 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Brief Description of the Figures

FIG. 1 shows the nucleotide (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of DR5. It is predicted that amino acids 1-51 (underlined) constitute the signal peptide (amino acid residues from about -51 to about -1 in SEQ ID NO:2); amino acids 52-184 constitute the extracellular domain (amino acid residues from about 1 to about 133 in SEQ ID NO:2); amino acids 185-208 (underlined) constitute the transmembrane domain (amino acid residues from about 134 to about 157 in SEQ ID NO:2); and amino acids 209-411 constitute the intracellular domain (amino acid residues from about 158 to about 360 in SEQ ID NO:2), of which amino acids 324-391 (italicized) constitute the death domain (amino acid residues from about 273 to about 340 in SEQ ID NO:2).

FIG. 2 shows the regions of similarity between the amino acid sequences of DR5 (HLYBX88), human tumor necrosis factor receptor 1 (h TNFR1) (SEQ ID NO:3), human Fas protein (SEQ ID NO:4), and the death domain containing receptor 3 (SEQ ID NO:5). The comparison was created with the Megalign program which is contained in the DNA Star suite of programs, using the Clustal method.

FIG. 3 shows an analysis of the DR5 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues about 62 to about 110, about 119 to about 164, about 224 to about 271, and about 275 to about 370 as depicted in Figure 1 correspond to the shown highly antigenic regions of the DR5 protein. These highly antigenic fragments in Figure 1 correspond to the following fragments, respectively, in SEQ ID NO:2: amino acid residues from about 11 to about 59, from about 68 to about 113, from about 173 to about 220, and from about 224 to about 319.

FIG. 4 shows the nucleotide sequences (HAPBU13R and HSBBU76R) of two cDNA molecules which are related to the nucleotide sequence shown in Figure 1 (SEQ ID NO:1).

FIG. 5A is a bar graph showing that overexpression of DR5 induced apoptosis in MCF7 human breast carcinoma cells. **FIG. 5B** is a bar graph showing that overexpression of DR5 induced apoptosis in human epitheloid carcinoma (Hela) cells.

FIG. 5C is a bar graph showing that DR5-induced apoptosis was blocked by caspase inhibitors, CrmA and z-VAD-fmk, but dominant negative FADD was without effect. **FIG. 5D** is an immunoblot showing that, like DR4, DR5 did not interact with FADD and TRADD *in vivo*. **FIG. 5E** is a bar graph showing that a dominant negative version of a newly identified FLICE-like molecule, FLICE2 (Vincenz, C. *et al., J. Biol. Chem. 272*:6578 (1997)), efficiently blocked DR5-induced apoptosis, while dominant negative FLICE had only partial effect under conditions it blocked. It also shows that TNFR-1 blocked apoptosis effectively.

FIG. 6A is an immunoblot showing that DR5-Fc (as well as DR4 and TRID) specifically bound TRAIL, but not the related cytotoxic ligand TNFα. The bottom panel of Fig. 6A shows the input Fc-fusions present in the binding assays. **FIG. 6B** is a bar graph showing that DR5-Fc blocked the ability of TRAIL to induce apoptosis . The data (mean ± SD) shown in Fig. 6B are the percentage of apoptotic nuclei among total nuclei counted (n=4). **FIG. 6C** is a bar graph showing that DR5-Fc had no effect on apoptosis TNFα-induced cell death under conditions where TNFR1-Fc completely abolished TNFα killing.

Detailed Description of the Preferred Embodiments

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a DR5 polypeptide having the amino acid sequence shown in FIG. 1 (SEQ ID NO:2), or a fragment of the polypeptide. The DR5 polypeptide of the present invention shares sequence homology with other known death domain containing receptors of the TNFR family including human TNFR- I, DR3 and Fas (FIG. 2). The nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) was obtained by sequencing cDNA clones such as HLYBX88, which was deposited on March 7, 1997 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number 97920. The deposited clone is contained in the pSport 1 plasmid (Life Technologies, Gaithersburg, MD).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art

for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Using the information provided herein, such as the nucleic acid sequence set out in SEQ ID NO:1, a nucleic acid molecule of the present invention encoding a DR5 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule of the invention has been identified in cDNA libraries of the following tissues: primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia, and human thymus stromal cells.

The determined nucleotide sequence of the DR5 cDNA of SEQ ID NO:1 contains an open reading frame encoding a protein of about 411 amino acid residues whose initiation codon is at position 130-132 of the nucleotide sequence shown in FIG. 1 (SEQ ID NO.1), with a leader sequence of about 51 amino acid residues. Of known members of the TNF receptor family, the DR5 polypeptide of the invention shares the greatest degree of homology with human TNFR1, FAS and DR3 polypeptides shown in Fig. 2, including significant sequence homology over multiple cysteine-rich domains. The homology DR5 shows to other death domain containing receptors strongly indicates that DR5 is also a death domain containing receptor with the ability to induce apoptosis. DR5 has also now been shown to bind TRAIL.

As indicated, the present invention also provides the mature form(s) of the DR5 protein of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or

more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide.

Therefore, the present invention provides a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in the host identified as ATCC Deposit No. 97920, and as shown in Figure 1 (SEQ ID NO:2). By the mature DR5 protein having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97920, is meant the mature form(s) of the DR5 protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the vector in the deposited host. As indicated below, the mature DR5 having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920, may or may not differ from the predicted "mature" DR5 protein shown in SEQ ID NO:2 (amino acids from about 1 to about 360) depending on the accuracy of the predicted cleavage site based on computer analysis.

Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res. 3*:271-286 (1985)) and von Heinje (*Nucleic Acids Res. 14*:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80%. von Heinje, *supra*. However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the predicted amino acid sequence of the complete DR5 polypeptide of the present invention was analyzed by a computer program ("PSORT"). *See,* K. Nakai and M. Kanehisa, *Genomics 14*:897-911 (1992). PSORT is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis by the PSORT program predicted the cleavage sites between amino acids 51 and 52 in Figure 1 (-1 and 1 in SEQ ID NO:2). Thereafter, the complete amino acid sequences were further analyzed by visual inspection, applying a simple form of the (-1,-3) rule of von Heinje. von Heinje, *supra*. Thus, the leader sequence for the DR5 protein is predicted to consist of amino acid residues from about 1 to about 51, underlined in Figure 1 (corresponding to about -51 to about 1 in SEQ ID NO:2), while the predicted mature DR5 protein

consists of residues from about 52 to about 411 in Figure 1 (corresponding to about 1 to about 360 in SEQ ID NO:2).

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DR5 DNA molecules comprising an open reading frame (ORF) shown in SEQ ID NO:1; DNA molecules comprising the coding sequence for the mature DR5 protein; and DNA molecules which comprise a sequence substantially different from those described above, but which, due to the degeneracy of the genetic code, still encode the DR5 protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the DR5 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97920 on March 7, 1997. In a further embodiment, nucleic acid molecules are provided that encode the mature DR5 polypeptide or the full length DR5 polypeptide lacking the N-terminal methionine. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 or the nucleotide sequence of the DR5 cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping by *in situ* hybridization with chromosomes, and for detecting expression of the DR5 gene in human tissue, for instance, by Northern blot analysis

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated DNA molecule having the nucleotide sequence of the deposited cDNA, the nucleotide sequence shown in SEQ ID NO:1, or the complementary strand thereto, is intended DNA fragments at least about 15 nt, and more preferably at least 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40, 50, 100, 150, 200, 250, 300, 400, or 500 nt in length. These fragments have numerous uses which include, but are not limited to, diagnostic probes and primers as discussed herein. Of course larger DNA fragments 500-1500 nt in length are also useful according to the present invention, as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNA or as shown in SEQ ID NO:1. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited DNA or the nucleotide sequence as shown in SEQ ID NO:1.

Preferred nucleic acid fragments of the present invention include, but are not limited to nucleic acid molecules encoding: a polypeptide comprising the DR5 extracellular domain (amino acid residues from about 52 to about 184 in FIG. 1 (from about 1 to about 133 in SEQ ID NO:2)); a polypeptide comprising the DR5 transmembrane domain (amino acid residues from about 185 to about 208 in FIG. 1 (from about 134 to about 157 in SEQ ID NO:2)); a polypeptide comprising the DR5 intracellular domain (amino acid residues from about 209 to about 411 in FIG. 1 (from about 158 to about 360 in SEQ ID NO:2)); and a polypeptide comprising the DR5 death domain (amino acid residues from about 324 to about 391 in FIG. 1 (from about 273 to about 340 in SEQ ID NO:2)). Since the location of these domains have been predicted by computer graphics, one of ordinary skill would appreciate that the amino acid residues constituting these domains may vary slightly (e.g., by about 1 to 15 residues) depending on the criteria used to define each domain.

Preferred nucleic acid fragments of the invention encode a full-length DR5 polypeptide lacking the nucleotides encoding the amino-terminal methionine (nucleotides 130-132 in SEQ ID NO:1) as it is known that the methionine is cleaved naturally and such sequences maybe useful in genetically engineering DR5 expression vectors. Polypeptides encoded by such polynucleotides are also contemplated by the invention.

Preferred nucleic acid fragments of the present invention further include nucleic acid molecules encoding epitope-bearing portions of the DR5 protein. In particular, such nucleic acid fragments of the present invention include, but are not limited to,

nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 62 to about 110 in Figure 1 (about 11 to about 59 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 119 to about 164 in Figure 1 (about 68 to about 113 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 224 to about 271 in Figure 1 (about 173 to about 220 in SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 275 to about 370 in Figure 1 (about 224 to about 319 in SEQ ID NO:2). The inventors have determined that the above polypeptide fragments are antigenic regions of the DR5 protein. Methods for determining other such epitope-bearing portions of the DR5 protein are described in detail below.

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NO:1 which have been determined from the following related cDNA clones: HAPBU13R (SEQ ID NO:6) and HSBBU76R (SEQ ID NO:7). The nucleotide sequences of HAPBU13R and HSBBU76R are shown in Figure 4.

Further, the invention includes a polynucleotide comprising any portion of at least about 30 nucleotides, preferably at least about 50 nucleotides, of SEQ ID NO:1 from residue 284 to 1,362, preferably from 284 to 681.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit No. 97920. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 or 80-150 nt, or the entire length of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the

reference polynucleotide (e.g., the deposited cDNA or the nucleotide sequence as shown in SEQ ID NO:1).

Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of the DR5 cDNA shown in Figure 1 (SEQ ID NO:1)), or to a complementary stretch of T (or U) resides, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode a DR5 polypeptide may include, but are not limited to, the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al., Cell 37*:767 -778(1984). As discussed below, other such fusion proteins include the DR5 receptor fused to Fc at the N- or C- terminus.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs, or derivatives of the DR5 receptor. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New

York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions which may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions, and deletions, which do not alter the properties and activities of the DR5 receptor or portions thereof. Also especially preferred in this regard are conservative substitutions.

Further embodiments of the invention include isolated nucleic acid molecules that are at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the amino terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions about 1 to about 360 in SEQ ID NO:2; (d) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920; (e) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920; (f) a nucleotide sequence that encodes the DR5 extracellular domain having the amino acid sequence at positions about 1 to about 133 in SEQ ID NO:2, or the DR5 extracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920; (g) a nucleotide sequence that encodes the DR5 transmembrane domain having the amino acid sequence at positions about 134 to about 157 of SEQ ID NO:2, or the DR5 transmembrane domain encoded by the cDNA contained in ATCC Deposit No. 97920; (h) a nucleotide sequence that encodes the DR5 intracellular domain having the amino acid sequence at positions about 158 to about 360 of SEQ ID NO:2, or the DR5 intracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920; (i) a nucleotide sequence that encodes the DR5 death domain domain having the amino acid sequence at positions about 273 to about 340 of SEQ ID NO:2, or the DR5 death domain encoded by the cDNA contained in ATCC Deposit No. 97920; and (j) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), or (i) above.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a DR5 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference

sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the DR5 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. The reference (query) sequence may be the entire DR5 nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) or any polynucleotide fragment as described herein.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 or to the nucleotide sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics 2*:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identiy are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends,

relative to the the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. A determination of whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of this embodiment. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignement of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequnce are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1, the nucleic acid sequence of the deposited cDNAs, or fragments thereof, irrespective of whether they encode a polypeptide having DR5 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having DR5 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having DR5 activity include, *inter alia:* (1) isolating the DR5 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location

of the DR5 gene, as described in Verma *et al., Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting DR5 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1, the nucleic acid sequence of the deposited cDNAs, or fragments thereof, which do, in fact, encode a polypeptide having DR5 protein activity. By "a polypeptide having DR5 activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the DR5 protein of the invention (either the full-length protein or, preferably, the mature protein), as measured in a particular biological assay. For example, DR5 protein activity can be measured using the cell death assays performed essentially as previously described (A.M. Chinnaiyan, *et al., Cell 81*:505-12 (1995); M.P. Boldin, *et al., J Biol Chem 270*:7795-8 (1995); F.C. Kischkel, *et al., EMBO 14*:5579-5588 (1995); A.M. Chinnaiyan, *et al., J Biol Chem 271*:4961-4965 (1996)) and as set forth in Example 5, below. In MCF7 cells, plasmids encoding full-length DR5 or a candidate death domain containing receptor are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR5 will exhibit apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al., Cell 85*:817-827 (1996); M. P. Boldin, *et al., Cell 85*:803-815 (1996); M. Tewari, *et al., J Biol Chem 270*:3255-60 (1995)), DR5-induced apoptosis is preferably blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately reeognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited cDNA, the nucleic acid sequence shown in SEQ ID NO:1, or fragments thereof, will encode a polypeptide "having DR5 protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, in many instances, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having DR5 protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Polynucleotide Assays

This invention is also related to the use of the DR5 polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DR5 associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of DR5 or a soluble form thereof, such as, for example, tumors or autoimmune disease.

Individuals carrying mutations in the DR5 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. (Saiki *et al., Nature 324*:163-166 (1986)). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding DR5 can be used to identify and analyze DR5 expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled DR5 RNA or alternatively, radiolabeled DR5 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or

without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al., Science 230*:1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and Sl protection or the chemical cleavage method (e.g., Cotton *et al., Proc. Natl Acad. Sci. USA 85*: 4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods which include, but are not limited to, hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA).

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

Vectors and Host Cells

The present invention also relates to vectors which include DNA molecules of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked

to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s)), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites for transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Such markers include, but are not limited to, dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors available to those of skill in the art.

Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host strain may be chosen which modulates the expression of the inserted gene sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically engineered polypeptide may be controlled. Furthermore, different host cells have characteristics and specific mechanisms for the translational and post-translational processing and modification (e.g., phosphorylation, cleavage) of proteins. Appropriate cell lines can be chosen to ensure the desired modifications and processing of the foeign protein expressed.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods

are described in many standard laboratory manuals, such as Davis *et al., Basic Methods in Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein (comprising the polypeptide joined via a peptide bond to a heterologous protein sequence (of a different protein)), and may include not only secretion signals but also additional heterologous functional regions. Such a fusion protein can be made by ligating polynucleotides of the invention and the desired nucleic acid sequence encoding the desired amino acid sequence to each other, by methods known in the art, in the proper reading frame, and expressing the fusion protein product by methods known in the art. Alternatively, such a fusion protein can be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Additionally, a region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and-thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when the Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as an antigen for immunizations. In drug discovery, for example, human proteins, such as the hIL5-receptor, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, 270:9459-9471 (1995).

The DR5 polypeptides can be recovered and purified from recombinant cell cultures by standard methods which include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography,

phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

DR5 polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of DR5. Among these are applications in treatment of tumors, resistance to parasites, bacteria and viruses, to induce proliferation of T-cells, endothelial cells and certain hematopoietic cells, to treat restenosis, graft vs. host disease, to regulate anti-viral responses and to prevent certain autoimmune diseases after stimulation of DR5 by an agonist. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are discussed further below.

DR5 Polypeptides and Fragments

The invention further provides an isolated DR5 polypeptide having the amino acid sequence encoded by the deposited cDNA, or the amino acid sequence in SEQ ID NO:2, or a polypeptide or peptide comprising a portion of the above polypeptides.

It will be recognized in the art that some amino acid sequence of DR5 can be varied without significant effect on the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Such areas will usually comprise residues which make up the ligand binding site or the death domain, or which form tertiary structures which affect these domains.

Thus, the invention further includes variations of the DR5 protein which show substantial DR5 protein activity or which include regions of DR5, such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U. *et al., Science 247:*1306-1310 (1990).

Thus, the fragment, derivative, or analog of the polypeptide of SEQ ID NO:2, or that encoded by the deposited cDNA, may be (i) one in which at least one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue(s), and more preferably at least one but less than ten conserved amino acid residues) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Of particular interest are substitutions of charged amino acids with another charged amino acids and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the DR5 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al., Clin Exp. Immunol. 2*:331-340 (1967); Robbins *et al., Diabetes 36*:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems 10*:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al., Nature 361*:266-268 (1993) describes certain mutations resulting in selective binding of TNF-alpha to only one of the two known types of TNF receptors. Thus, the DR5 receptor of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions

Aromatic	Phenylalanine
	Tryptophan
	Tyrosine
Hydrophobic	Leucine
	Isoleucine
	Valine
Polar	Glutamine
	Asparagine
Basic	Arginine
	Lysine
	Histidine
Acidic	Aspartic Acid
	Glutamic Acid
Small	Alanine
	Serine
	Threonine
	Methionine
	Glycine

Amino acids in the DR5 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science 244*:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for

biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al., J. Mol. Biol. 224*:899-904 (1992) and de Vos *et al. Science 255*:306-312 (1992)).

The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell. For example, a recombinantly produced version of the DR5 polypeptide can be substantially purified by the one-step method described in Smith and Johnson, *Gene 67*:31-40 (1988).

The polypeptides of the present invention also include the polypeptide encoded by the deposited cDNA including the leader; the mature polypeptide encoded by the deposited the cDNA minus the leader (i.e., the mature protein); a polypeptide comprising amino acids about - 51 to about 360 in SEQ ID NO:2; a polypeptide comprising amino acids about - 50 to about 360 in SEQ ID NO:2; a polypeptide comprising amino acids about 1 to about 360 in SEQ ID NO:2; a polypeptide comprising the extracellular domain; a polypeptide comprising the transmembrane domain; a polypeptide comprising the intracellular domain; a polypeptide comprising the extracellular and intracellular domains with all or part of the transmembrane domain deleted; and a polypeptide comprising the death domain; as well as polypeptides which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98%, or 99% identical to the polypeptides described above, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a DR5 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the DR5 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may

be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figure 1 (SEQ ID NO:2), the amino acid sequence encoded by deposited cDNA clones, or fragments thereof, can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the

percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of this embodiment. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence. For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequnce are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The polypeptide of the present invention could be used as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art.

The present inventors have discovered that the DR5 polypeptide is a 411 residue protein exhibiting three main structural domains. First, the ligand binding domain was identified within residues from about 52 to about 184 in FIG. 1 (amino acid residues from about 1 to about 133 in SEQ ID NO:2). Second, the transmembrane domain was identified within residues from about 185 to about 208 in FIG. 1 (amino acid residues from about 134 to about 157 in SEQ ID NO:2). Third, the intracellular domain was identified within residues from about 209 to about 411 in FIG. 1 (amino acid residues from about 158 to about 360 in SEQ ID NO:2). Importantly, the intracellular domain includes a death domain at residues from about 324 to about 391 (amino acid residues from about 273 to about 340 in SEQ ID NO:2).

Further preferred fragments of the polypeptide shown in FIG. 1 include the mature protein from residues about 52 to about 411 (amino acid residues from about 1 to about 360 in SEQ ID NO:2), and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain.

The invention further provides DR5 polypeptides encoded by the deposited cDNA clone including the leader and DR5 polypeptide fragments selected from the mature protein, the extracellular domain, the transmembrane domain, the intracellular domain, the death domain, and all combinations thereof.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide described herein. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al., Proc. Natl. Acad. Sci. USA 81*:3998- 4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A., "Antibodies That React With Predetermined Sites on Proteins," *Science 219:*660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al., Cell 37*:767-778 (1984) at 777. Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine, and most preferably between at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DR5-specific antibodies include: a polypeptide comprising amino acid

residues from about 62 to about 110 in Figure 1 (about 11 to about 59 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 119 to about 164 in Figure 1 (about 68 to about 113 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 224 to about 271 in Figure 1 (about 173 to about 220 in SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 275 to about 370 in Figure 1 (about 224 to about 319 in SEQ ID NO:2). As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DR5 protein.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Hougthen, R.A., "General Method for the Rapid Solid-Phase Synthesis of Large Numbers of Peptides: Specificity of Antigen-Antibody Interaction at the Level of Individual Amino Acids," *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Hougthen *et al.* (1986).

As one of skill in the art will appreciate, DR5 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al., Nature 331*:84- 86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR5 protein or protein fragment alone (Fountoulakis *et al., J Biochem 270*:3958-3964 (1995)).

Polypeptide Assays

The present invention also relates to diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR5 protein, or the soluble form thereof, in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR5, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors, for example. Assay techniques that can be used to determine levels of a protein, such as a DR5 protein of the present invention, or a soluble form thereof, in a sample derived from a host are

well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis, and ELISA assays.

Assaying DR5 protein levels in a biological sample can occur using any art-known method. By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source containing DR5 receptor protein or mRNA. Preferred for assaying DR5 protein levels in a biological sample are antibody-based techniques. For example, DR5 protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen, M. *et al., J. Cell. Biol. 101:*976-985 (1985); Jalkanen, M. *et al., J. Cell. Biol. 105:*3087-3096 (1987)). Other antibody-based methods useful for detecting DR5 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as glucose oxidase, radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Therapeutics

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol. 51*:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem. 57*:505-518 (1988); Old, L.J., *Sci. Am. 258*:59-75 (1988); Fiers, W., *FEBS Lett. 285*:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors, including the DR5 of the present invention. Cells which express the DR5 polypeptide and are believed to have a potent cellular response to DR5 ligands include primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia, and human thymus stromal cells. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis (programmed cell death) is a physiological mechanism

involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Krammer, P.H. *et al., Curr. Opin. Immunol.* 6:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostrate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation; graft vs. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myelodysplastic syndromes (such as aplastic anemia), ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of DR5 ligand, analog or an agonist capable of increasing DR5 mediated signaling. Preferably, DR5 mediated signaling is increased to treat a disease wherein decreased apoptosis or decreased cytokine and adhesion molecule expression is exhibited. An agonist can include soluble forms of DR5 and monoclonal antibodies directed against the DR5 polypeptide.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the, DR5 polypeptide an effective amount of an antagonist capable of decreasing DR5 mediated signaling. Preferably, DR5 mediated signaling is decreased to treat a disease wherein increased apoptosis or NFkB expression is exhibited. An antagonist can include soluble forms of DR5 and monoclonal antibodies directed against the DR5 polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit

apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation. For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds (antagonists) which inhibit activation of the receptor polypeptide of the present invention by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds

to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem. 267*:4304-4307(1992).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR5 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DR5 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor, neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonist s include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and -amyloid peptide. (*Science 267*:1457-1458 (1995)). Further preferred agonist include polyclonal and monoclonal antibodies raised against the DR5 polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., *et al., Proc. Natl. Acad. Sci. USA 88*:9292-9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem. 267* (7):4304-4307 (1992) See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *ElB*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1, LMP-1,* African swine fever virus *LMW5-HL,* and Herpesvirus yl 34.5), calpain inhibitors, cysteine protease inhibitors,

and tumor promoters (such as PMA, Phenobarbital, and alpha-Hexachlorocyclohexane).

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem. 56*:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al., Nucleic Acids Research 6*:3073 (1979); Cooney *et al., Science 241*:456 (1988); and Dervan *et al., Science 251*:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide.

In one embodiment, the DR5 antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the DR5 antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others know in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding DR5, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or a constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, *Nature* 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., *Cell* 22:787-797 (1980), the herpes thymidine promoter (Wagner et al., *Proc. Natl. Acad. Sci.* U.S.A. 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster, et al., *Nature* 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a DR5 gene. However,

absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded DR5 antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a DR5 RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Potential antagonists acccording to the invention also include catalytic RNA, or a ribozyme (See, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al, *Science* 247:1222-1225 (1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy DR5 mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature* 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of DR5 (FIG. 1). Preferrably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the DR5 mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. Since ribozymes, unlike antisense molecules are catalytic, a lower intracellular concentration is required for efficiency.

Further antagonist according to the present invention include soluble forms of DR5, i.e., DR5 fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DR5 mediated signaling by competing with the cell surface DR5 for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. These may be expressed as

monomers, but, are preferably expressed as dimers or trimers, since these have been shown to be superior to monomeric forms of soluble receptor as antagonists, e.g., IgGFc-TNF receptor family fusions. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med. 182*:1395-1401 (1995)).

The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab, and $F(ab')_2$ fragments) which are capable of binding an antigen. Fab, Fab', and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med. 24*:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of standard methods using DR5 immunogens of the present invention. As indicated, such DR5 immunogens include the full length DR5 polypeptide (which may or may not include the leader sequence) and DR5 polypeptide fragments such as the ligand binding domain, the transmembrane domain, the intracellular domain and the death domain.

Antibodies of the invention can be used in methods known in the art relating to the localization and activity of the polypeptide sequences of the invention, e.g., for imaging these polypeptides, measuring levels thereof in appropriate physiological samples, etc. The antibodies also have use in immunoassays and in therapeutics as agonists and antagonists of DR5.

Proteins and other compounds which bind the DR5 domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature 340*:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. *et al., Cell 75*:791-803 (1993); Zervos, A.S. *et al., Cell 72*:223-232 (1993)). Preferably, the yeast two-hybrid system is used according to the present invention to capture compounds which bind to either the DR5 ligand binding domain or to the DR5 intracellular domain. Such compounds are good candidate agonist and antagonist of the present invention.

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, DR5 ligands, TRAIL, TNF-α, lymphotoxin-α

(LT-α, also known as TNF-β), LT-β (found in complex heterotrimer LT-α2-β), FasL, CD40, CD27, CD30, 4-lBB, OX40 and nerve growth factor (NGF). An example of an assay that can be performed to determine the ability of DR5 and derivatives (including fragments) and analogs thereof to bind TRAIL is described below in Example 6.

Representative therapeutic applications of the present invention are discussed in-more detail below. The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of $CD4^+$ T-lymphocytes. Recent reports estimate the daily loss of $CD4^+$ T cells to be between 3.5 X 10^7 and 2 X 10^9 cells (Wei X. *et al., Nature 373*:117-122 (1995)). One cause of $CD4^+$ T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis. Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS 8*:1197-1213 (1994) ; Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol. 6*:605-615(1995); Muro-Cacho, C.A. *et al., J. Immunol. 154*:5555-5566 (1995)). Furthermore, apoptosis and $CD4^+$ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., *et al., Nature 373*:441-444 (1995); Gougeon, M.L., *et al., AIDS Res. Hum. Retroviruses 9*:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. *et al., AIDS Res. Hum. Retroviruses 9*:553-563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (Badley, A.D. *et al., J. Virol. 70*:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D *et al., J. Virol. 70*:199-206 (1996)). Thus, by the invention, a method for treating HIV^+ individuals is provided which involves administering an antagonist of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive

regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. Agonists of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the DR5 polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues.

DR5 antagonists may be useful for treating inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In addition, due to lymphoblast expression of DR5, soluble DR5 agonist or antagonist mABs may be used to treat this form of cancer. Further, soluble DR5 or neutralizing mABs may be used to treat various chronic and acute forms of inflammation such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

Modes of Administration

The agonist or antagonists described herein can be administered *in vitro*, *ex vivo*, or *in vivo* to cells which express the receptor of the present invention. By administration of an "effective amount" of an agonist or antagonist is intended an amount of the compound that is sufficient to enhance or inhibit a cellular response to a TNF-family ligand and include polypeptides. In particular, by administration of an "effective amount" of an agonist or antagonists is intended an amount effective to enhance or inhibit DR5 mediated apoptosis. Of course, where it is desired for apoptosis is to be enhanced, an agonist according to the present invention can be co-administered with a TNF-family ligand. One of ordinary skill will appreciate that effective amounts of an agonist or antagonist can be determined empirically and may be employed in pure form or in pharmaceutically acceptable salt, ester or prodrug form. The agonist or antagonist may be administered in compositions in combination with one or more pharmaceutically acceptable excipients (i.e., carriers).

It will be understood that, when administered to a human patient, the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgement. The

specific therapeutically effective dose level for any particular patient will depend upon factors well known in the medical arts.

As a general proposition, the total pharmaceutically effective amount of DR5 polypeptide administered parenterally per dose will be in the range of about 1 µg/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the DR5 agonists or antagonists is typically administered at a dose rate of about 1 µg/kg/hour to about 50 µg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Dosaging may also be arranged in a patient specific manner to provide a predetermined concentration of an agonist or antagonist in the blood, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, on the order of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml.

Pharmaceutical compositions are provided comprising an agonist or antagonist (including DR5 polynucleotides and polypeptides of the invention) and a pharmaceutically acceptable carrier or excipient, which may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), bucally, or as an oral or nasal spray. Importantly, by co-administering an agonist and a TNF-family ligand, clinical side effects can be reduced by using lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. In a specific embodiment, "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly humans. Nonlimiting examples of suitable pharmaceutical carriers according to this embodiment are provided in "Remington's Pharmaceutical Sciences" by E.W. Martin, and include sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered

intravenously. Saline solutions and aqueous dextrose and glyceral solutions can be employed as liquid carriers, particularly for injectable solutions.

The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

In addition to soluble DR5 polypeptides, DR5 polypeptide containing the transmembrane region can also be used when appropriately solubilized by including detergents, such as CHAPS or NP-40, with buffer.

Chromosome assays

The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA and/or polynucleotides herein disclosed is used to clone genomic DNA of a DR5 gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA is then used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bp. For a review of this technique, see Verma *et al., Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man*, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Example 1

Expression and Purification in E. coll

The DNA sequence encoding the mature DR5 protein in the deposited cDNA clone (ATCC No. 97920) is amplified using PCR oligonucleotide primers specific to the amino terminal sequences of the DR5 protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The following primers are used for expression of DR5 extracellular domain in *E. coli*: The 5' primer has the sequence 5'-CGCCCATGGAGTCT GCTCTGATCAC-3' (SEQ ID NO:8) and contains the underlined NcoI site; and the 3' primer has the sequence 5'-CGCAAGCTTTTAGCCTGATTC TTTGTGGAC-3' (SEQ ID NO:9) and contains the underlined HindIII site.

The restriction sites are convenient to restriction enzyme sites in the bacterial expression vector pQE60, which are used for bacterial expression in this example. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, and a ribosome binding site ("RBS").

The amplified DR5 DNA and the vector pQE60 both are digested with NcoI and HindIII and the digested DNAs are then ligated together. Insertion of the DR5 protein DNA into the restricted pQE60 vector places the DR5 protein coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-

frame with an initiating AUG appropriately positioned for translation of DR5 protein.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing DR5 protein, is available commercially from Qiagen, *supra*.

Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR, and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600nm ("OD600") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lac*I repressor. Cells subsequently are incubated further for 3 to 4 hours.

Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS at a concentration of 95 µ/ml.

Example 2

Expression in Mammalian Cells

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the

transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g. RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular signals can also be used (e.g. the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human Hela 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. Co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The dihydrofolate reductase (DHFR) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy *et al., Biochem. J. 227*:277-279 (1991); Bebbington *et al., Bio/Technology 10:*169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

The expression vectors pCl and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molecular and Cellular Biology* 5:438-447 (March 1985)), plus a fragment of the CMV-enhancer (Boshart *et al., Cell 41*:521-530 (1985)). Multiple cloning sites, e.g. with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of the DR5 polypeptide. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter.

Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids, can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate (MTX). The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., *J. Biol. Chem. 253*:1357-1370 (1978); Hamlin, J. L. and Ma, C., *Biochem. et Biophys. Acta 1097*:107-143 (1990); Page, M. J. and Sydenham, M. A. 1991, *Biotechnology 9*:64-68(1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains, for expressing the gene of interest, the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen et al., *Molecular and Cellular Biology* 5:438-447(March 1985), plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart et al., *Cell 41*:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Xba I, and Asp718. Behind these cloning sites the plasmid contains the 3′ intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for expression, e.g., the human ß-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the DR5 polypeptide in a regulated way in mammalian cells (Gossen, M., & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992). For the polyadenylation of the mRNA, other signals, e.g., from the human growth hormone or globin genes, can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418, or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphates by procedures known in the art.

The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. The 5' primer containing the underlined BamHI site, a Kozak sequence, and an AUG start codon, has the following sequence: 5'-CGCGGATCCGCCATCATGGAACAACGGGGACAGAAC-3' (SEQ ID NO:10). The 3' primer, containing the underlined Asp718 site, has the following sequence: 5'-CGCGGTACCTTAGGACATGGCAGAGTC-3' (SEQ ID NO:11).

The amplified fragment is digested with the endonuclease BamHI and Asp718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSVneo using the lipofectin method (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of metothrexate plus 1 mg/ml G418. After about 10-14 days, single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 µM, 2 µM, 5 µM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 µM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Cloning and Expression in COS Cells

The expression plasmid, pDR5-HA, is made by cloning a cDNA encoding the soluble extracellular domain of the DR5 protein into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.). The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation

in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 and a polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. A DNA fragment encoding the extracelluar domain of the DR5 polypeptide and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson et al., *Cell 37*: 767 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is as follows. The DR5 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of DR5 *in E coli*. To facilitate detection, purification and characterization of the expressed DR5, one of the primers contains a hemagglutinin tag ("HA tag") as described above.

Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined BamHI site has the following sequence: 5'-CGCGGATCCGCCATCATGGAACAACGGGGACAGAAC-3' (SEQ ID NO:10). The 3' primer, containing the underlined Asp718 restriction sequence has the following sequence:5'-CGCGGTACCTTAGCCTGATTCTTTTGGAC-3' (SEQ ID NO:12).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with BamHI and Asp718 and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the extracellular domain of the DR5 polypeptide

For expression of recombinant DR5, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook et al., *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of DR5 by the vector.

Expression of the DR5-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow *et al.*,

Antibodies: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing ^{35}S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and the lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson *et al.*, cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

The primer sets used for expression in this example are compatible with pC4 used for CHO expression in this example, pcDNAI/Amp for COS expression in this example, and pA2 used for baculovirus expression in the following example. Thus, for example, the complete DR5 encoding fragment amplified for CHO expression could also be ligated into pcDNAI/Amp for COS expression or pA2 for baculovirus expression.

Example 3

Cloning and expression of the soluble extracellular domain of DR5 in a baculovirus expression system

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding the complete protein, including its naturally associated signal sequence, into a baculovirus to express the DR5 protein, using standard methods, such as those described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedron promoter of the *Autograph californica nuclear polyhedrosis virus* (ACMNPV) followed by convenient restriction sites. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak *Drosophila* promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide. Many other baculovirus vectors could be used in place of pA2, such as pAc373, pVL941 and pAcIM1 provided, as one skilled in the art would readily appreciate, that construction provides appropriately located signals for transcription, translation,

secretion, and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described, for example, in Luckow *et al., Virology 170*:31-39 (1989).

The cDNA sequence encoding the soluble extracellular domain of DR5 protein in the deposited clone (ATCC No. 97920) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer for DR5 has the sequence 5'-CGCGGATCCGCCATCATGGA ACAACGGGGACAGAAC-3' (SEQ ID NO:10) containing the underlined BamHI restriction enzyme site. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DR5 provides an efficient cleavage signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer for DR5 has the sequence 5'-CGCGGTACCTTAGCCT GATTCTTTGTGGAC-3' (SEQ ID NO:12) containing the underlined Asp718 restriction followed by nucleotides complementary to the DR5 nucleotide sequence in FIG. 1, followed by the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.) The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated "F1."

The plasmid is digested with the restriction enzymes Bam HI and Asp718 and optionally can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). The vector DNA is designated herein "V1."

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. *E. coli* HB101 cells, or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells, are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human DR5 using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies containing the DR5 gene fragment will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac DR5.

5 μg of the plasmid pBac DR5 is co-transfected with 1.0 μg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA",

Pharmingen, San Diego, CA.), using the lipofectin method described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987). 1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac DR5 are mixed in a sterile well of a microliter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours, the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days ,the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg, MD) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, MD, pages 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e..g, Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 µl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later, the supernatants of these culture dishes are harvested and then they are stored at 4°C. The recombinant virus is called V- DR5.

To verify expression of the DR5 gene, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V- DR5 at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later, the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Gaithersburg, MD). If radiolabeled proteins are desired, 42 hours later, 5 µCi of ^{35}S-methionine and 5 µCi

^{35}S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of

the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature protein and thus the cleavage point and length of the secretory signal peptide.

Example 4

Tissue distribution of DR5 gene expression

Northern blot analysis was carried out to examine DR5 gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR5 protein (SEQ ID NO:1) was labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe was purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe was then used to examine various human tissues for DR5 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) were obtained from Clontech (Palo Alto, CA) and examined with labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots were mounted and exposed to film at -70°C overnight. The films were developed according to standard procedures. Expression of DR5 was detected in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, uterus, small intestine, colon, peripheral blood leukocytes (PBLs), lymph node, bone marrow, and fetal liver.

Expression of DR5 was also assessed by Northern blot in the following cancer cell lines, HL60 (promyelocytic leukemia), Hela cell S3, K562 (chronic myelogeneous leukemia), MOLT4 (lymphoblast leukemia), Raji (Burkitt's lymphoma), SW480 (colorectal adenocarcinoma), A549 (lung carcinoma), and G361 (melanoma), and was detected in all of the cell lines tested.

Example 5

DR5 Induced Apoptosis in Mammalian Cells

Overexpression of Fas/APO-1 and TNFR-1 in mammalian cells mimics receptor activation (M. Muzio *et al.*, *Cell 85*: 817-827 (1996); M. P. Boldin *et al.*, *Cell 85*:803-815 (1996)). Thus, this system was utilized to study the functional role of DR5 in inducing apoptosis. This example demonstrates that overexpression of DR5

induced apoptosis in both MCF7 human breast carcinoma cells and in human epitheloid carcinoma (Hela) cells.

Experimental Design

Cell death assays were performed essentially as previously described (A.M. Chinnaiyan, *et al., Cell 81*:505-12 (1995); M.P. Boldin, *et al., J Biol Chem 270*: 7795-8 (1995); F.C. Kischkel, *et al., EMBO 14*:5579-5588 (1995); A.M. Chinnaiyan, *et al., J Biol Chem 271*: 4961-4965 (1996)). Briefly, MCF-7 human breast carcinoma clonal cell lines and Hela cells were co-transfected with vector, DR5, DR5Δ (52-411), or TNFR-1, together with a beta-galactosidase reporter construct.

MCF7 and Hela cells were transfected using the lipofectamine procedure (GIBCO-BRL), according to the manufacturer's instructions. 293 cells were transfected using $CaPO_4$ precipitation. Twenty-four hours following transfection, cells were fixed and stained with X-Gal as previously described (A.M. Chinnaiyan, *et al., Cell 81*:505-12 (1995); M.P. Boldin, *et al., J Biol Chem 270*:7795-8 (1995); F.C. Kischkel, *et al., EMBO 14*:5579-5588 (1995)), and examined microscopically. The data (mean±SD) presented in Figure 5 represents the percentage of round, apoptotic cells as a function of total beta-galactosidase positive cells (n=3). Overexpression of DR5 induced apoptosis in both MCF7 (Fig. 5A) and Hela cells (Fig. 5B).

MCF7 cells were also transfected with a DR5 expression construct in the presence of z-VAD-fmk (20μl)(Enzyme Systems Products, Dublin, CA) or co-transfected with a three-fold excess of CrmA (M. Tewari *et al., J Biol Chem 270*:3255-60 (1995)), or FADD-DN expression construct, or vector alone. The data presented in Fig. 5C shows that apoptosis induced by DR5 was attenuated by caspase inhibitors ,but not by dominant negative FADD.

As depicted in Fig. 5D, DR5 did not associate with FADD or TRADD in vivo. 293 cells were co-transfected with the indicated expression constructs using calcium phosphate precipitation. After transfection (at 40 hours), cell lysates were prepared and immunoprecipitated with Flag M2 antibody affinity gel (IBI, Kodak), and the presence of FADD or myc-tagged TRADD (myc-TRADD) was detected by immunoblotting with polyclonal antibody to FADD or horseradish peroxidase (HRP) conjugated antibody to myc (BMB)(Baker, S.J. *et al.*, Oncogene 12:1 (1996); Chinnaiyan, A.M. *et al., Science 274*:990 (1996)).

As depicted in Fig. 5E, FLICE 2-DN blocks DR5-induced apoptosis. 293 cells were co-transfected with DR5 or TNFR-1 expression construct and a fourfold excess of CrmA, FLICE-DN, FLICE 2-DN, or vector alone in the presence of a beta-

galactosidase reported construct as indicated. Cells were stained and examined 25-30 hours later.

Results

Overexpression of DR5, induced apoptosis in both MCF7 human breast carcinoma cells (Fig. 5A) and in human epitheloid carcinoma (Hela) cells (Fig. 5B). Most of the transfected cells displayed morphological changes characteristic of cells undergoing apoptosis (Earnshaw, W.C., *Curr. Biol.* 7:337 (1995)), becoming rounded, condensed and detaching from the dish. Deletion of the death domain abolished killing ability. Like DR4, DR5-induced apoptosis was blocked by caspase inhibitors, CrmA and z-VAD-fmk, but dominant negative FADD was without effect (Fig. 5C). Consistent with this, DR5 did not interact with FADD and TRADD *in vivo* (Fig. 5D). A dominant negative version of a newly identified FLICE-like molecule, FLICE2 (Vincenz, C. *et al., J. Biol. Chem. 272*:6578 (1997)), efficiently blocked DR5-induced apoptosis, while dominant negative FLICE had only partial effect under conditions it blocked. TNFR-1 induced apoptosis effectively (Fig. 5E). Taken together, the evidence suggests that DR5 engages an apoptotic program that involves activation of FLICE2 and downstream caspases, but is independent of FADD.

Example 6

The Extracellular Domain of DR5 Binds the Cytotoxic Ligand--TRAIL, and Blocks TRAIL-Induced Apoptosis

As discussed above, TRAIL/Apo2L is a cytotoxic ligand that belongs to the tumor necrosis factor (TNF) ligand family and induces rapid cell death of many transformed cell lines, but not normal tissues, despite its death domain containing receptor, DR4, being expressed on both cell types. This example shows that the present receptor, DR5, also binds TRAIL.

Given the similarity of the extracellular ligand binding cysteine-rich domains of DR5 and DR4, the present inventors theorized that DR5 would also bind TRAIL. To confirm this, the soluble extracellular ligand binding domains of DR5 were expressed as fusions to the Fc portion of human immunoglobulin (IgG).

As shown in Fig. 6A, DR5-Fc specifically bound TRAIL, but not the related cytotoxic ligand TNFα. In this experiment, the Fc-extracellular domains of DR5, DR4, TRID, or TNFR1 and the corresponding ligands were prepared and binding assays performed as described in Pan *et al., Science 276*:111 (1997). The respective Fc-fusions were precipitated with protein G-Sepharose and co-precipitated soluble

ligands were detected by immunoblotting with anti-Flag (Babco) or anti-myc-HRP (BMB). The bottom panel of Fig. 6A shows the input Fc-fusions present in the binding assays.

Additionally, DR5-Fc blocked the ability of TRAIL to induce apoptosis (Fig. 6B). MCF7 cells were treated with soluble TRAIL (200 ng/ml) in the presence of equal amounts of Fc-fusions or Fc alone. Six hours later, cells were fixed and examined as desribed in Pan et al., *Id.* The data (mean ± SD) shown in Fig. 6B are the percentage of apoptotic nuclei among total nuclei counted (n=4).

Finally, DR5-Fc had no effect on apoptosis TNFα-induced cell death under conditions where TNFR1-Fc completely abolished TNFα killing (Fig 6C). MCF7 cells were treated with TNFα (40 ng/ml; Genentech, Inc.) in the presence of equal amounts of Fc-fusions or Fc alone. Nuclei were stained and examined 11-15 hours later.

The new identification of DR5 as a receptor for TRAIL adds further complexity to the biology of TRAIL-initiated signal transduction.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosures of all patents, patent applications, and publications referred to herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising amino acids from about -51 to about 360 in SEQ ID NO:2;

(b) a nucleotide sequence encoding a polypeptide comprising amino acids from about -50 to about 360 in SEQ ID NO:2;

(c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 360 in SEQ ID NO:2;

(d) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(e) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(f) a nucleotide sequence encoding the DR5 extracellular domain;

(g) a nucleotide sequence encoding the DR5 transmembrane domain;

(h) a nucleotide sequence encoding the DR5 intracellular domain;

(i) a nucleotide sequence encoding the DR5 death domain; and

(j) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), or (i) above.

2. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1.

3. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence encoding the DR5 polypeptide having the amino acid sequence in SEQ ID NO:2.

4. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1 encoding the mature DR5 polypeptide having the amino acid sequence in SEQ ID NO:2.

5. The nucleic acid molecule of claim 1, wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 97920.

6. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence encoding the DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920.

7. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920.

8. An isolated nucleic acid molecule comprising a polynucleotide sequence which hybridizes under stringent hybridization conditions to a polynucleotide sequence having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j) of claim 1, wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only adenosine nucleotides or of only thymidine nucleotides.

9. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a DR5 polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), (f), (g), (h), or (i) of claim 1.

10. The isolated nucleic acid molecule of claim 9, which encodes an epitope-bearing portion of a DR5 polypeptide selected from the group consisting of: a polypeptide comprising amino acid residues from about 11 to about 59 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 68 to about 113 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 173 to about 220 in SEQ ID NO:2; and a polypeptide comprising amino acid residues from about 224 to about 319 in SEQ ID NO:2.

11. The isolated nucleic acid molecule of claim 1, which encodes the DR5 extracellular domain.

12. The isolated nucleic acid molecule of claim 1, which encodes the DR5 transmembrane domain.

13. The isolated nucleic acid molecule of claim 1, which encodes the DR5 intracellular domain

14. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

15. A recombinant vector produced by the method of claim 14.

16. A method of making a recombinant host cell comprising introducing an isolated nucleic acid molecule of claim 1 into a host cell.

17. A recombinant host cell produced by the method of claim 16.

18. A recombinant method for producing a DR5 polypeptide, comprising culturing the recombinant host cell of claim 17 under conditions such that said polypeptide is expressed and recovering said polypeptide.

19. An isolated DR5 polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) amino acids from about -51 to about 360 in SEQ ID NO:2;

(b) amino acids from about -50 to about 360 in SEQ ID NO:2;

(c) amino acids from about 1 to about 360 in SEQ ID NO:2;

(d) the amino acid sequence of the DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(e) the amino acid sequence of the mature DR5 polypeptide having the amino acid encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(f) the amino acid sequence of the DR5 extracellular domain;

(g) the amino acid sequence of the DR5 transmembrane domain;

(h) the amino acid sequence of the DR5 intracellular domain;

(i) the amino acid sequence of the DR5 death domain;

(j) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j).

20. An isolated polypeptide comprising an epitope-bearing portion of the DR5 protein, wherein said portion is selected from the group consisting of: a polypeptide comprising amino acid residues from about amino acid residues from about 11 to about 59 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 68 to about 113 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 173 to about 220 in SEQ ID NO:2; and a polypeptide comprising amino acid residues from about 224 to about 319 in SEQ ID NO:2.

21. An isolated antibody that binds specifically to a DR5 polypeptide of claim 19.

22. An isolated nucleic acid molecule comprising a polynucleotide having a sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the nucleotide sequence of clone HAPBU13R (SEQ ID NO:6);

(b) the nucleotide sequence of clone HSBBU76R (SEQ ID NO:7);

(c) the nucleotide sequence of a portion of the sequence shown in Figure 1 (SEQ ID NO:1) wherein said portion comprises at least 50 contiguous nucleotides from nucleotide 284 to 1,362; and

(d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b) or (c) above.

23. An isolated nucleic acid molecule comprising a polynucleotide encoding a DR5 polypeptide wherein, except for at least one conservative amino acid substitution, said polypeptide has a sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising amino acids from about -51 to about 360 in SEQ ID NO:2;

(b) a nucleotide sequence encoding a polypeptide comprising amino acids from about -50 to about 360 in SEQ ID NO:2;

(c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 360 in SEQ ID NO:2;

(d) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(e) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(f) a nucleotide sequence encoding theDR5 extracellular domain;

(g) a nucleotide sequence encoding the DR5 transmembrane domain;

(h) a nucleotide sequence encoding the DR5 intracellular domain;

(i) a nucleotide sequence encoding the DR5 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted;

(j) a nucleotide sequence encoding the DR5 death domain; and

(k) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j).

24. An isolated DR5 polypeptide wherein, except for at least one conservative amino acid substitution, said polypeptide has a sequence selected from the group consisting of:

(a) amino acids from about -51 to about 360 in SEQ ID NO:2;

(b) amino acids from about -50 to about 360 in SEQ ID NO:2;

(c) amino acids from about 1 to about 360 in SEQ ID NO:2;

(d) the amino acid sequence of the DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(e) the amino acid sequence of the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(f) the amino acid sequence of the DR5 receptor extracellular domain;

(g) the amino acid sequence of the DR5 receptor transmembrane domain;

(h) the amino acid sequence of the DR5 receptor intracellular domain;

(i) the amino acid sequence of the DR5 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted;

(j) the amino acid sequence of the DR5 receptor death domain; and

(k) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j).

25. A pharmaceutical composition comprising the polypeptide of claim 19 and a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising the antibody of claim 21 and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising the polypeptide of claim 24 and a pharmaceutically acceptable carrier.

28. A fusion protein comprising the polypeptide of claim 19 fused to a heterologeous polypeptide.

29. The isolated nucleic acid of claim 8, wherein said nucleic acid encodes a protein which is able to be bound by an antibody to a DR5 polypeptide, wherein said polypeptide has the amino acid sequence in SEQ ID NO:2

30. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 8 into a vector.

31. A recombinant vector produced by the method of claim 30.

32. A method of making a recombinant host cell comprising introducing an isolated nucleic acid molecule of claim 8 into a host cell.

33. A recombinant host cell produced by the method of claim 32.

34. A recombinant method for producing a polypeptide, comprising culturing the recombinant host cell of claim 33 under conditions such that said polypeptide is expressed and recovering said polypeptide.

Death Domain Containing Receptor 5

Abstract

The present invention relates to novel Death Domain Containing Receptor-5 (DR5) proteins which are members of the tumor necrosis factor (TNF) receptor family, and have now been shown to bind TRAIL. In particular, isolated nucleic acid molecules are provided encoding the human DR5 proteins. DR5 polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying antagonists and antagonists of DR5 activity.

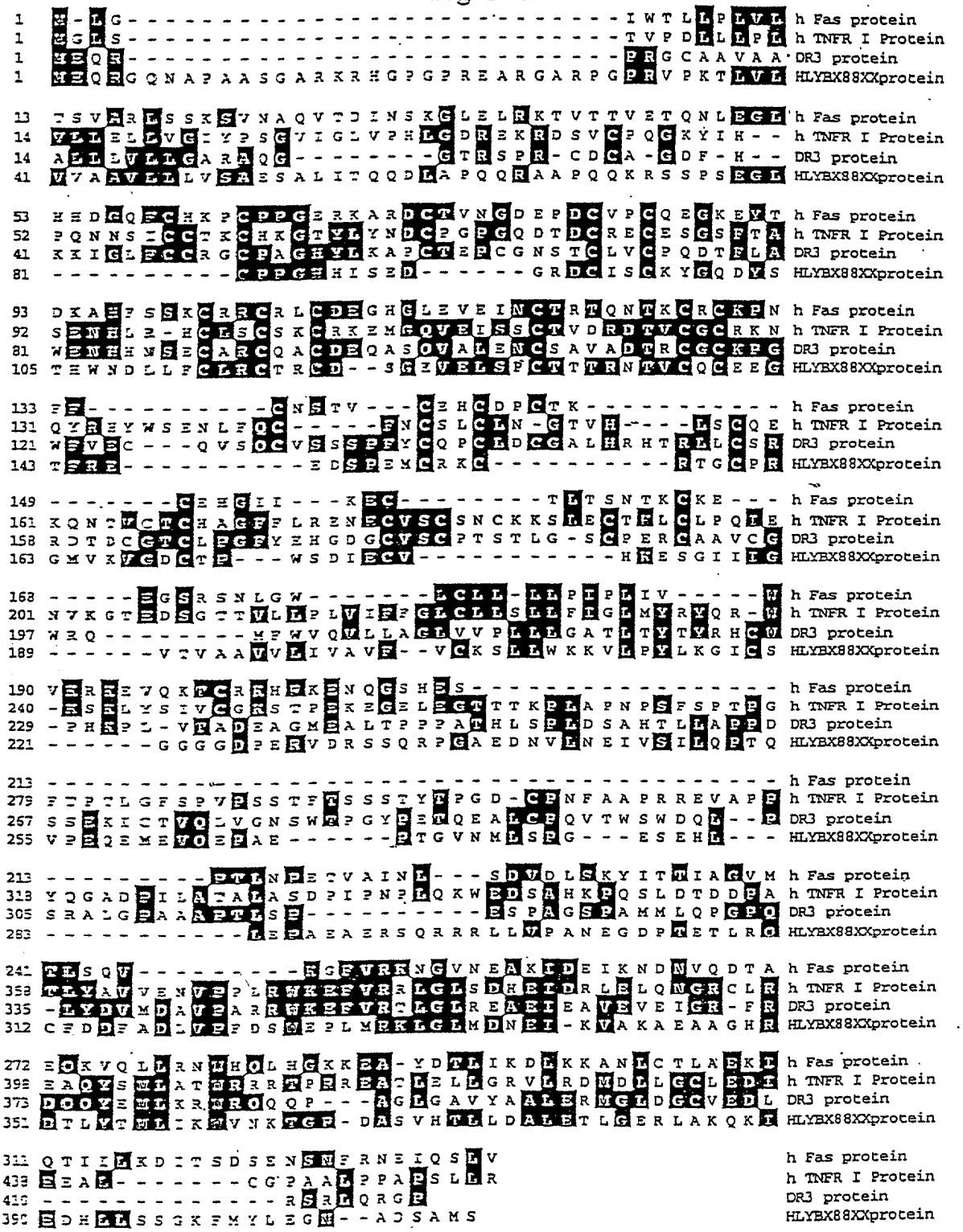
Figure 1

```
          10                  30                  50
CACGCGTCCGCGGGCGCGGCCGGAGAACCCCGCAATCTTTGCGCCCACAAAATACACCGA
          70                  90                 110
CGATGCCCGATCTACTTTAAGGGCTGAAACCCACGGGCCTGAGAGACTATAAGAGCGTTC
         130                 150                 170
CCTACCGCCATGGAACAACGGGGACAGAACGCCCCGGCCGCTTCGGGGGCCCGGAAAAGG
           M   E   Q   R   G   Q   N   A   P   A   A   S   G   A   R   K   R
         190                 210                 230
CACGGCCCAGGACCCAGGGAGGCGCGGGGAGCCAGGCCTGGGCCCCGGGTCCCCAAGACC
H   G   P   G   P   R   E   A   R   G   A   R   P   G   P   R   V   P   K   T
         250                 270                 290
CTTGTGCTCGTTGTCGCCGCGGTCCTGCTGTTGGTCTCAGCTGAGTCTGCTCTGATCACC
L   V   L   V   V   A   A   V   L   L   L   V   S   A   E   S   A   L   I   T
         310                 330                 350
CAACAAGACCTAGCTCCCCAGCAGAGAGCGGCCCCACAACAAAAGAGGTCCAGCCCCTCA
Q   Q   D   L   A   P   Q   Q   R   A   A   P   Q   Q   K   R   S   S   P   S
         370                 390                 410
GAGGGATTGTGTCCACCTGGACACCATATCTCAGAAGACGGTAGAGATTGCATCTCCTGC
E   G   L   C   P   P   G   H   H   I   S   E   D   G   R   D   C   I   S   C
         430                 450                 470
AAATATGGACAGGACTATAGCACTCACTGGAATGACCTCCTTTTCTGCTTGCGCTGCACC
K   Y   G   Q   D   Y   S   T   H   W   N   D   L   L   F   C   L   R   C   T
         490                 510                 530
AGGTGTGATTCAGGTGAAGTGGAGCTAAGTCCCTGCACCACGACCAGAAACACAGTGTGT
R   C   D   S   G   E   V   E   L   S   P   C   T   T   T   R   N   T   V   C
         550                 570                 590
CAGTGCGAAGAAGGCACCTTCCGGGAAGAAGATTCTCCTGAGATGTGCCGGAAGTGCCGC
Q   C   E   E   G   T   F   R   E   E   D   S   P   E   M   C   R   K   C   R
         610                 630                 650
ACAGGGTGTCCCAGAGGGATGGTCAAGGTCGGTGATTGTACACCCTGGAGTGACATCGAA
T   G   C   P   R   G   M   V   K   V   G   D   C   T   P   W   S   D   I   E
         670                 690                 710
TGTGTCCACAAAGAATCAGGCATCATCATAGGAGTCACAGTTGCAGCCGTAGTCTTGATT
C   V   H   K   E   S   G   I   I   I   G   V   T   V   A   A   V   V   L   I
         730                 750                 770
GTGGCTGTGTTTGTTTGCAAGTCTTTACTGTGGAAGAAAGTCCTTCCTTACCTGAAAGGC
V   A   V   F   V   C   K   S   L   L   W   K   K   V   L   P   Y   L   K   G
         790                 810                 830
ATCTGCTCAGGTGGTGGTGGGGACCCTGAGCGTGTGGACAGAAGCTCACAACGACCTGGG
I   C   S   G   G   G   G   D   P   E   R   V   D   R   S   S   Q   R   P   G
         850                 870                 890
GCTGAGGACAATGTCCTCAATGAGATCGTGAGTATCTTGCAGCCCACCCAGGTCCCTGAG
A   E   D   N   V   L   N   E   I   V   S   I   L   Q   P   T   Q   V   P   E
         910                 930                 950
CAGGAAATGGAAGTCCAGGAGCCAGCAGAGCCAACAGGTGTCAACATGTTGTCCCCCGGG
Q   E   M   E   V   Q   E   P   A   E   P   T   G   V   N   M   L   S   P   G
         970                 990                1010
GAGTCAGAGCATCTGCTGGAACCGGCAGAAGCTGAAAGGTCTCAGAGGAGGAGGCTGCTG
E   S   E   H   L   L   E   P   A   E   A   E   R   S   Q   R   R   R   L   L
        1030                1050                1070
```

Figure 1 (continued)

```
GTTCCAGCAAATGAAGGTGATCCCACTGAGACTCTGAGACAGTGCTTCGATGACTTTGCA
V  P  A  N  E  G  D  P  T  E  T  L  R  Q  C  F  D  D  F  A
      1090                1110                1130
GACTTGGTGCCCTTTGACTCCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAAT
D  L  V  P  F  D  S  W  E  P  L  M  R  K  L  G  L  M  D  N
      1150                1170                1190
GAGATAAAGGTGGCTAAAGCTGAGGCAGCGGGCCACAGGGACACCTTGTACACGATGCTG
E  I  K  V  A  K  A  E  A  A  G  H  R  D  T  L  Y  T  M  L
      1210                1230                1250
ATAAAGTGGGTCAACAAAACCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATGCCTTG
I  K  W  V  N  K  T  G  R  D  A  S  V  H  T  L  L  D  A  L
      1270                1290                1310
GAGACGCTGGGAGAGAGACTTGCCAAGCAGAAGATTGAGGACCACTTGTTGAGCTCTGGA
E  T  L  G  E  R  L  A  K  Q  K  I  E  D  H  L  L  S  S  G
      1330                1350                1370
AAGTTCATGTATCTAGAAGGTAATGCAGACTCTGCCATGTCCTAAGTGTGATTCTCTTCA
K  F  M  Y  L  E  G  N  A  D  S  A  M  S  *
      1390                1410                1430
GGAAGTGAGACCTTCCCTGGTTTACCTTTTTTCTGGAAAAAGCCCAACTGGACTCCAGTC
      1450                1470                1490
AGTAGGAAAGTGCCACAATTGTCACATGACCGGTACTGGAAGAAACTCTCCCATCCAACA
      1510                1530                1550
TCACCCAGTGGATGGAACATCCTGTAACTTTTCACTGCACTTGGCATTATTTTTATAAGC
      1570                1590
TGAATGTGATAATAAGGACACTATGGAAAAAAAAAAAAAA
```

Figure 2



Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly.

Figure 3

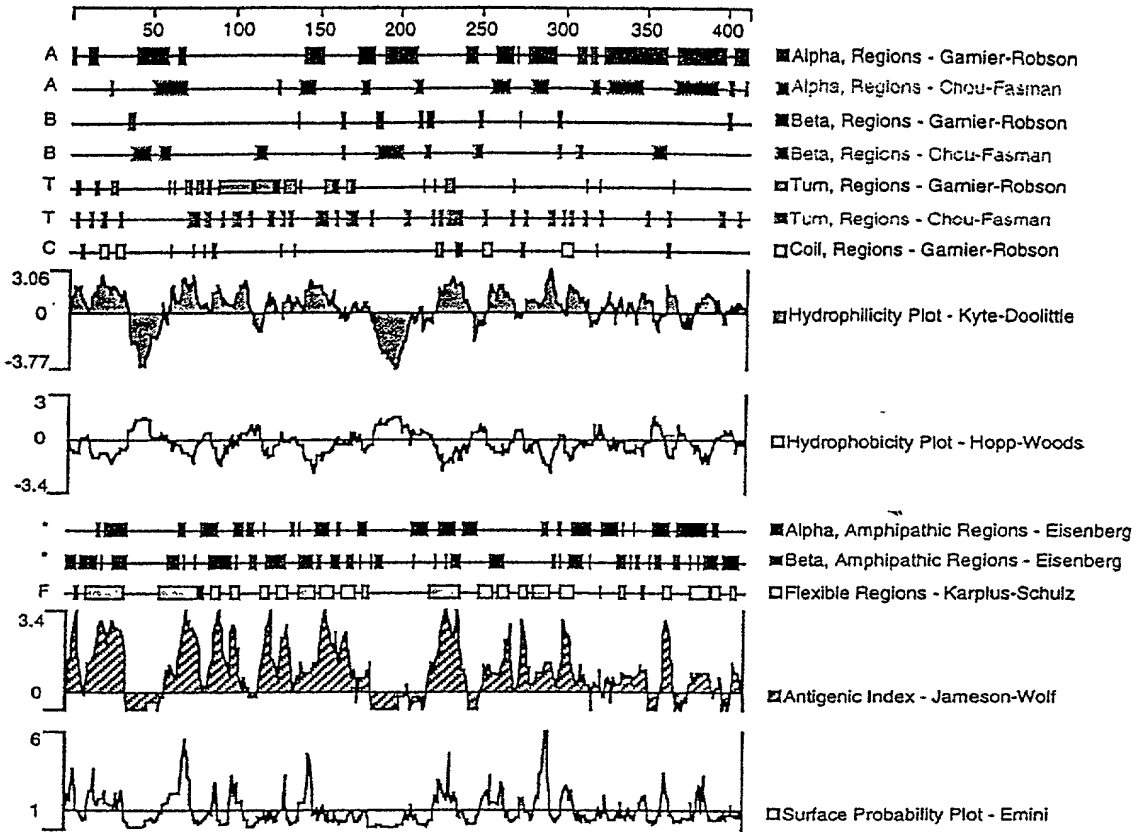

50
100
150
200
250
300
350
400
A
A
B
B
T
T
C
Alpha, Regions - Garnier-Robson
Alpha, Regions - Chou-Fasman
Beta, Regions - Garnier-Robson
Beta, Regions - Chou-Fasman
Turn, Regions - Garnier-Robson
Turn, Regions - Chou-Fasman
Coil, Regions - Garnier-Robson
3.06
0
-3.77
Hydrophilicity Plot - Kyte-Doolittle
3
0
-3.4
Hydrophobicity Plot - Hopp-Woods
Alpha, Amphipathic Regions - Eisenberg
Beta, Amphipathic Regions - Eisenberg
F
Flexible Regions - Karplus-Schulz
3.4
0
Antigenic Index - Jameson-Wolf
6
1
Surface Probability Plot - Emini

Figure 4

HAPBU13R

```
  1 AATTCGGCAC AGCTCTTCAG GAAGTCAGAC CTTCCCTGGT TTACCTTTTT
 51 TCTGGAAAAA GCCCAACTGG GACTCCAGTC AGTAGGAAAG TGCCACAATT
101 GTCACATGAC CGGTACTGGA AGAAACTCTC CCATCCAACA TCACCCAGTG
151 GNATGGGAAC ACTGATGAAC TTTTCACTGC ACTTGGCATT ATTTTTGTNA
201 AGCTGAATGT GATAATAAGG GCACTGATGG AAATGTCTGG ATCATTCCGG
251 TTGTGCGTAC TTTGAGATTT GNGTTTGGGG ATGTNCATTG TGTTTGACAG
301 CACTTTTTTN ATCCCTAATG TNAAATGCNT NATTTGATTG TGANTTGGGG
351 GTNAACATTG GTNAAGGNTN CCCNTNTGAC ACAGTAGNTG GTNCCCGACT
401 TANAATNGNN GAANANGATG NATNANGAAC CTTTTTTTGG GTGGGGGGGT
451 NNCGGGGCAG TNNAANGNNG NCTCCCCAGG TTTGGNGTNG CAATNGNGGA
501 ANNNTGG
```

HSBBU76R

```
  1 TTTTTTTTGT AGATGGATCT TACAATGTAG CCCAAATAAA TAAATAAAGC
 51 ATTTACATTA GGATAAAAAA GTGCTGTGAA AACAATGACA TCCCAAACCA
101 AATCTCAAAG TACGCACAAA CGGAATGATC CAGACATTTC CATAGNGTCC
151 TTATTATCAC ATTCAGCTTA TAAAANTAAT GCCAAGTGCA GTGAAAAGTT
201 ACAGGATGTT CCATCCACTG GGTGGATT
```

Figure 5

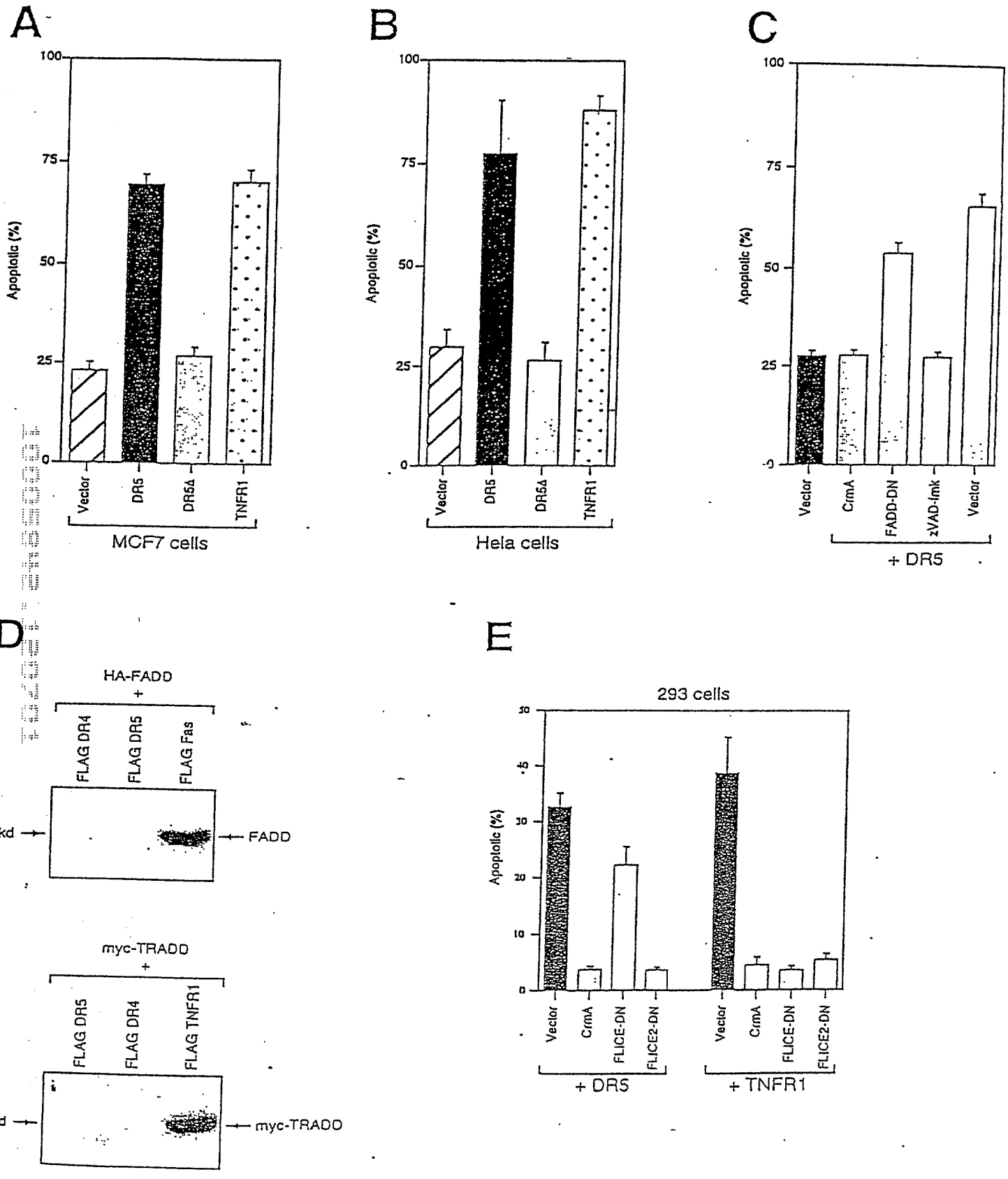

A
Apoptotic (%)
100
75
50
25
0
Vector
DR5
DR5Δ
TNFR1
MCF7 cells
B
Apoptotic (%)
Vector
DR5
DR5Δ
TNFR1
Hela cells
C
Apoptotic (%)
Vector
CrmA
FADD-DN
zVAD-fmk
Vector
+ DR5
D
HA-FADD
+
FLAG DR4
FLAG DR5
FLAG Fas
29 kd
FADD
myc-TRADD
+
FLAG DR5
FLAG DR4
FLAG TNFR1
45 kd
myc-TRADD
E
293 cells
Apoptotic (%)
50
40
30
20
10
0
Vector
CrmA
FLICE-DN
FLICE2-DN
Vector
CrmA
FLICE-DN
FLICE2-DN
+ DR5
+ TNFR1

Figure 6

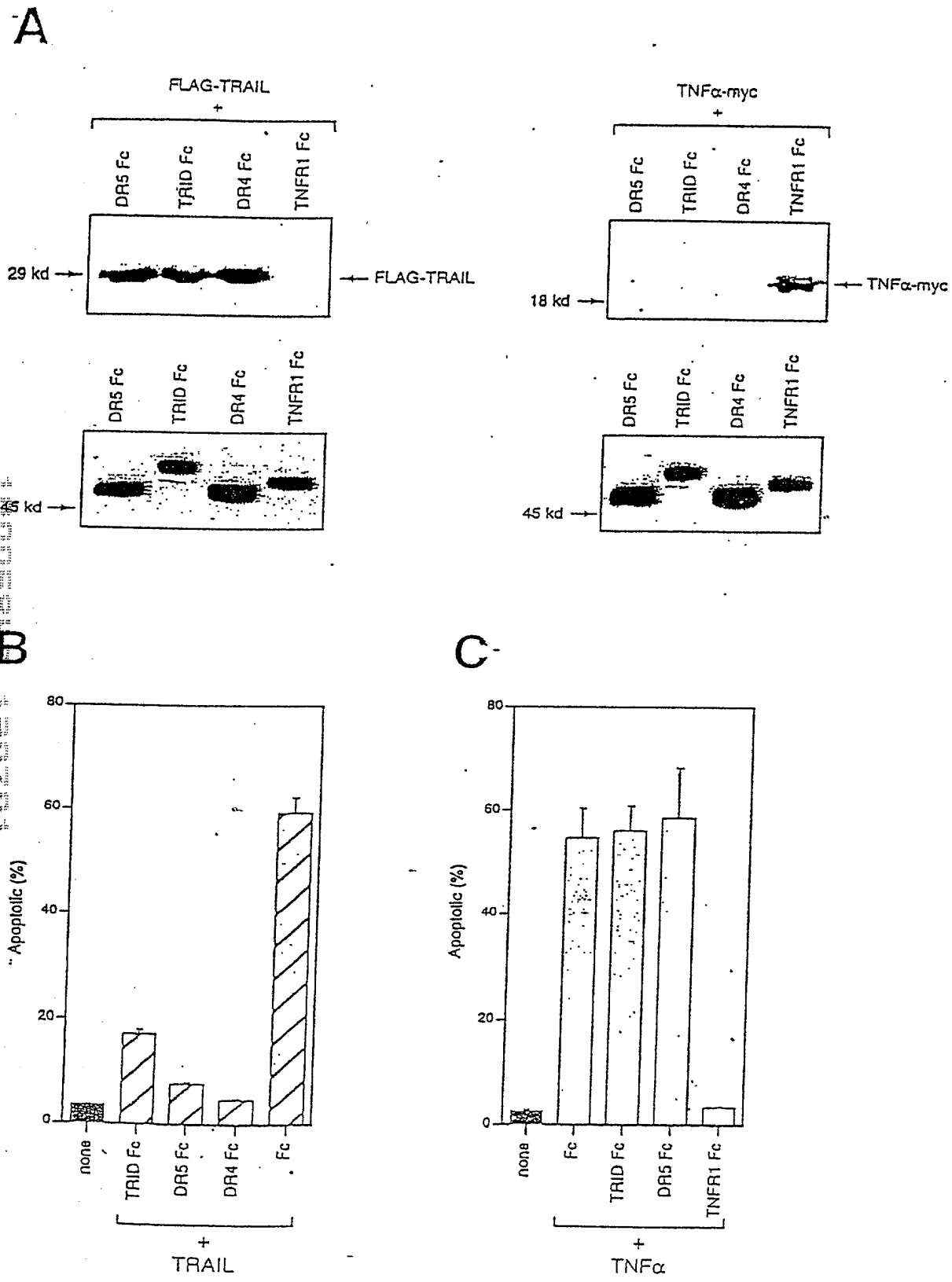

A
FLAG-TRAIL
+
DR5 Fc
TRID Fc
DR4 Fc
TNFR1 Fc
29 kd
FLAG-TRAIL
45 kd
TNFα-myc
18 kd
TNFα-myc
B
Apoptotic (%)
80
60
40
20
0
none
TRID Fc
DR5 Fc
DR4 Fc
Fc
TRAIL
C
Apoptotic (%)
Fc
TNFR1 Fc
TNFα

EXHIBIT C

US006642358B1

(12) **United States Patent**
Rauch et al.

(10) **Patent No.: US 6,642,358 B1**
(45) **Date of Patent: Nov. 4, 2003**

(54) **RECEPTOR THAT BINDS TRAIL**

(75) Inventors: **Charles Rauch**, Bainbridge Island, WA (US); **Henning Walczak**, Seattle, WA (US)

(73) Assignee: **Immunex Corporation**, Seattle, WA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/578,392**

(22) Filed: **May 25, 2000**

Related U.S. Application Data

(60) Division of application No. 08/883,036, filed on Jun. 26, 1997, now Pat. No. 6,072,047, which is a continuation-in-part of application No. 08/869,852, filed on Jun. 4, 1997, now abandoned, which is a continuation-in-part of application No. 08/829,536, filed on Mar. 28, 1997, now abandoned, which is a continuation-in-part of application No. 08/815,255, filed on Mar. 12, 1997, now abandoned, which is a continuation-in-part of application No. 08/799,861, filed on Feb. 13, 1997, now abandoned.

(51) **Int. Cl.**[7] **C07K 14/00**; C07K 21/04; C12N 15/00

(52) **U.S. Cl.** **530/350**; 530/300; 435/69.1; 435/7.1; 435/320.1; 435/325; 435/252.3; 435/172.3; 536/23.1; 536/23.5

(58) **Field of Search** 530/300, 350; 435/69.1, 7.1, 320.1, 325, 252.3, 172.3; 536/23.1, 23.5

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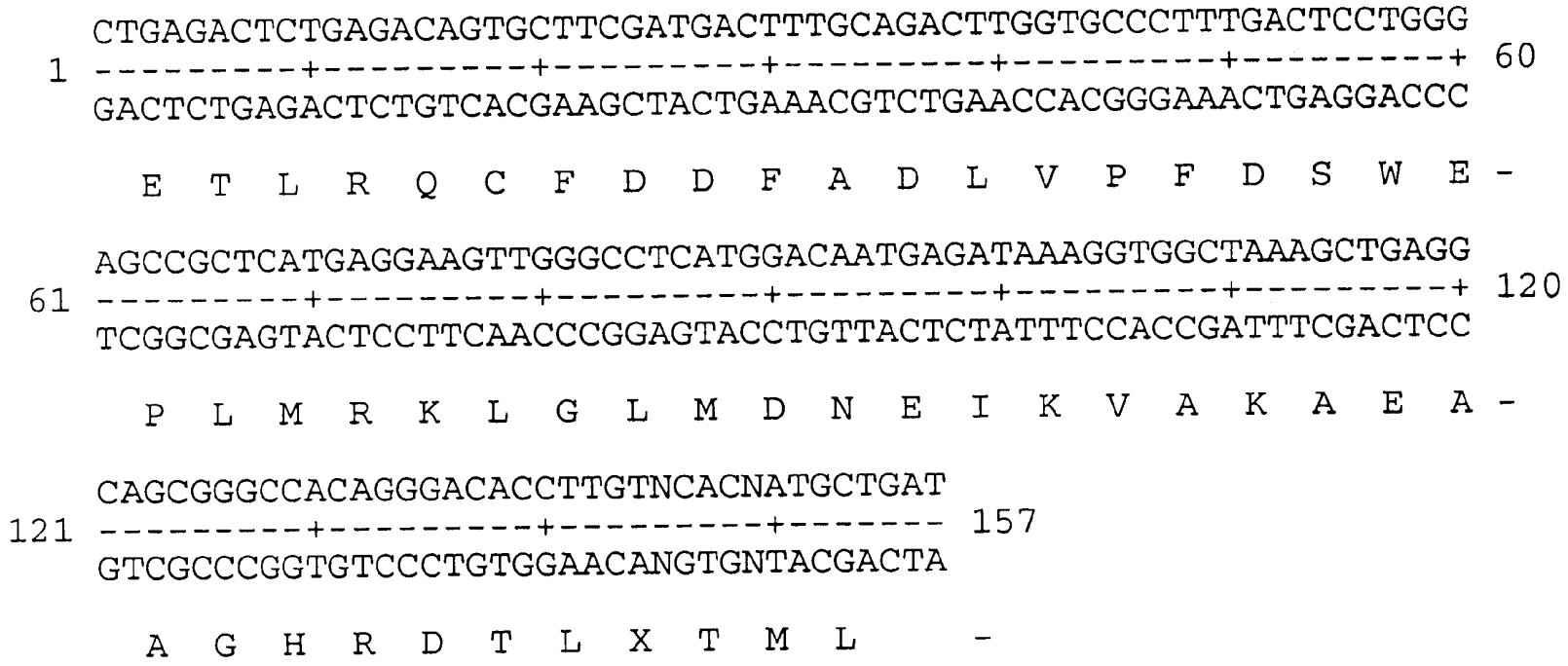
Primary Examiner—Gary Kunz
Assistant Examiner—Nirmal Basi
(74) *Attorney, Agent, or Firm*—Julie K. Smith; Kathryn A. Anderson; Stuart Watt

(57) **ABSTRACT**

A protein designated TRAIL receptor binds the protein known as TNF-Related Apoptosis-Inducing Ligand (TRAIL). The TRAIL receptor finds use in purifying TRAIL or inhibiting activities thereof. Isolated DNA sequences encoding TRAIL-R polypeptides are provided, along with expression vectors containing the DNA sequences, and host cells transformed with such recombinant expression vectors. Antibodies that are immunoreactive with TRAIL-R are also provided.

41 Claims, 3 Drawing Sheets

FIGURE 1



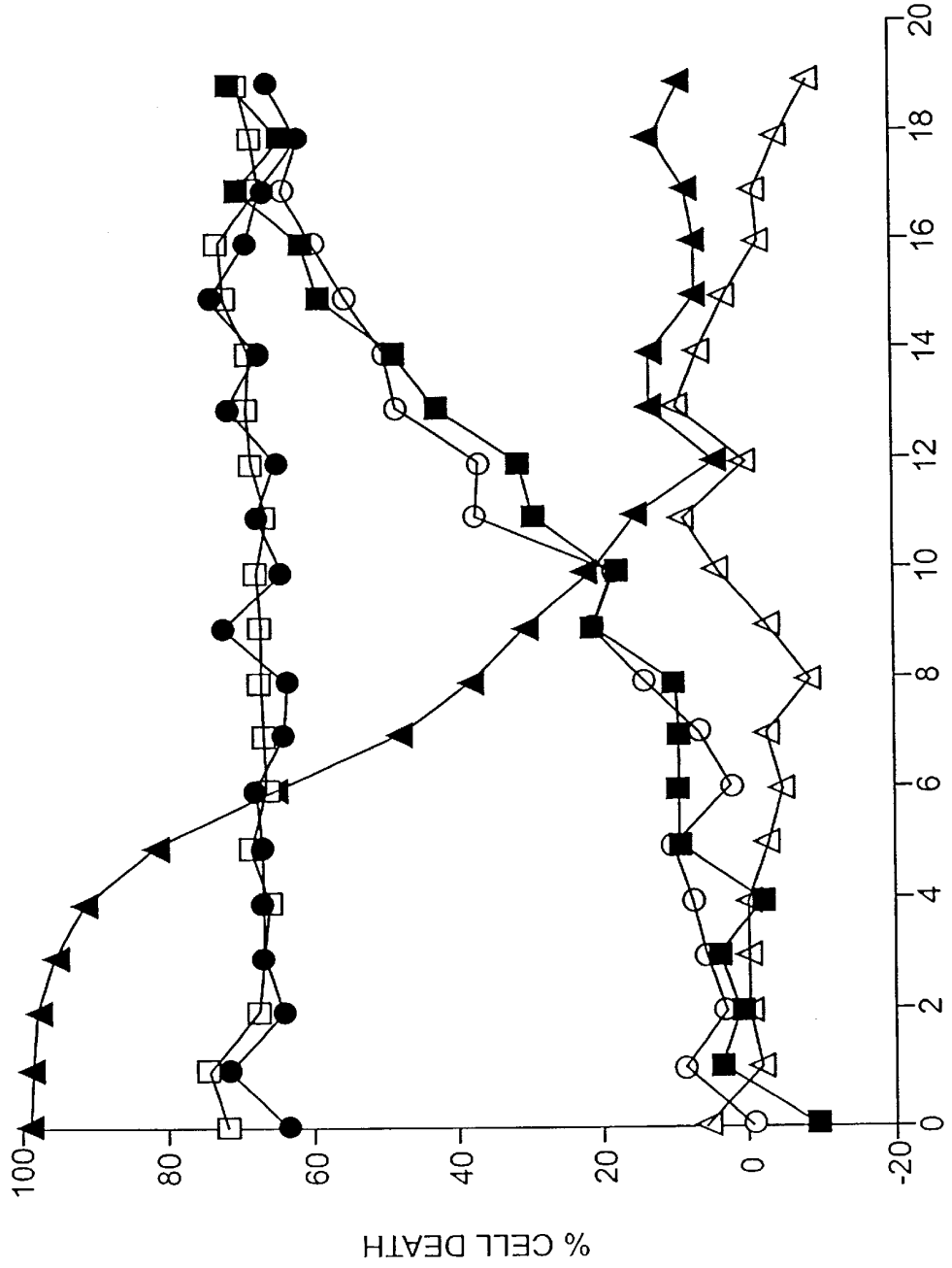

% CELL DEATH
100
80
60
40
20
0
-20
0
2
4
6
8
10
12
14
16
18
20
DILUTION (2^n)

FIG. 2

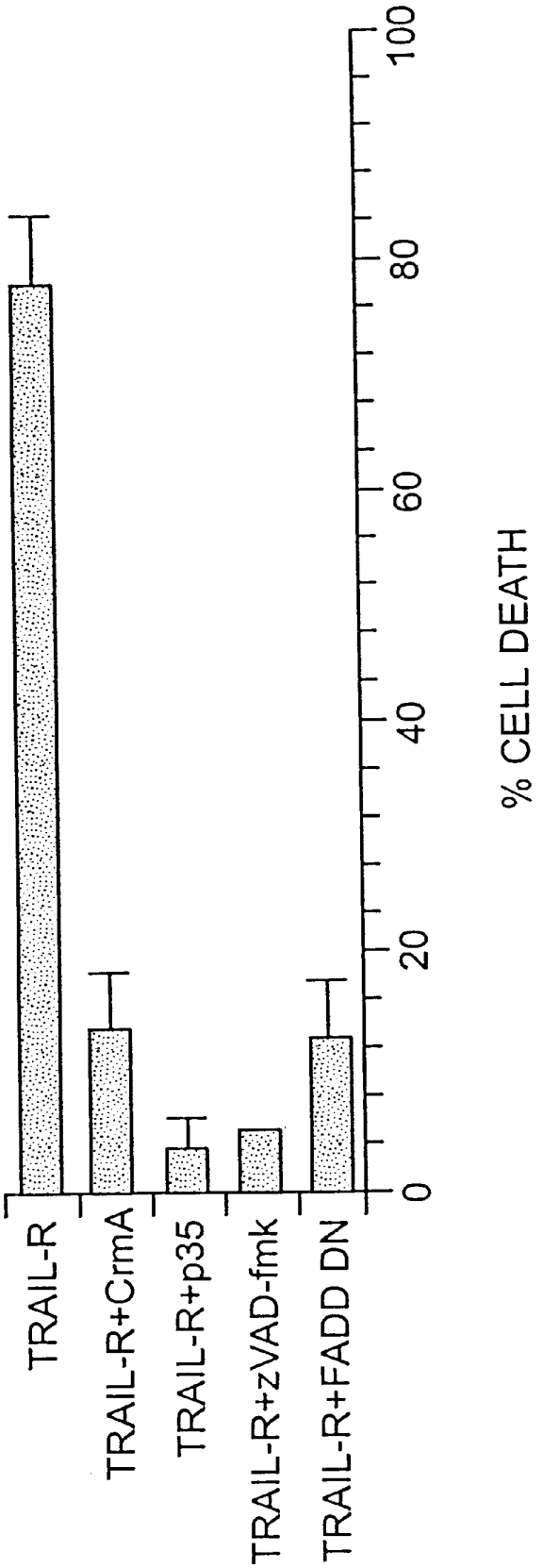

% CELL DEATH
0
20
40
60
80
100
TRAIL-R
TRAIL-R+CrmA
TRAIL-R+p35
TRAIL-R+zVAD-fmk
TRAIL-R+FADD DN

FIG. 3

RECEPTOR THAT BINDS TRAIL

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of application Ser. No. 08/883,036, filed Jun. 26, 1997, now U.S. Pat. No. 6,072,047, which is a continuation-in-part of application Ser. No. 08/869,852, filed Jun. 4, 1997, now abandoned, which is a continuation-in-part of application Ser. No. 08/829,536, filed Mar. 28, 1997, now abandoned, which is a continuation-in-part of application Ser. No. 08/815,255, filed Mar. 12, 1997, now abandoned, which is a continuation-in-part of application Ser. No. 08/799,861, filed Feb. 13, 1997, now abandoned.

BACKGROUND OF THE INVENTION

A protein known as TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands (Wiley et al., *Immunity,* 3:673–682, 1995). TRAIL has demonstrated the ability to induce apoptosis of certain transformed cells, including a number of different types of cancer cells as well as virally infected cells (PCT application WO 97/01633 and Wiley et al., supra).

Identification of receptor protein(s) that bind TRAIL would prove useful in further study of the biological activities of TRAIL. However, prior to the present invention, no receptor for TRAIL had been reported.

SUMMARY OF THE INVENTION

The present invention is directed to a novel protein designated TRAIL receptor (TRAIL-R), which binds to a protein known as TNF-related apoptosis-inducing ligand (TRAIL). DNA encoding TRAIL-R, and expression vectors comprising such DNA, are provided. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. Antibodies that are immunoreactive with TRAIL-R are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** presents the nucleotide sequence of a human TRAIL receptor DNA fragment, SEQ ID NO:3 as well as the amino acid sequence encoded thereby SEQ ID NO:4. This DNA fragment is described in Example 3.

FIG. **2** presents the results of the assay described in example 7. In the assay, a soluble TRAIL-R/Fc fusion protein blocked TRAIL-induced apoptosis of Jurkat cells.

FIG. **3** presents the results of the experiment described in example 8. The indicated compounds were demonstrated to inhibit apoptosis of cells expressing TRAIL receptor.

DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL receptor (TRAIL-R) is provided herein. TRAIL-R binds to the cytokine designated TNF-related apoptosis-inducing ligand (TRAIL). Certain uses of TRAIL-R flow from this ability to bind TRAIL, as discussed further below. TRAIL-R finds use in inhibiting biological activities of TRAIL, or in purifying TRAIL by affinity chromatography, for example.

The nucleotide sequence of the coding region of a human TRAIL receptor DNA is presented in SEQ ID NO:1. The

amino acid sequence encoded by the DNA sequence of SEQ ID NO:1 is shown in SEQ ID NO:2. This sequence information identifies the TRAIL receptor protein as a member of the tumor necrosis factor receptor (TNF-R) family of receptors (reviewed in Smith et al., *Cell* 76:959–962, 1994). The extracellular domain contains cysteine rich repeats; such motifs have been reported to be important for ligand binding in other receptors of this family. TRAIL-R contains a so-called "death domain" in the cytoplasmic region; such domains in certain other receptors are associated with transduction of apoptotic signals. These and other features of the protein are discussed in more detail below.

TRAIL-R protein or immunogenic fragments thereof may be employed as immunogens to generate antibodies that are immunoreactive therewith. In one embodiment of the invention, the antibodies are monoclonal antibodies.

A human TRAIL-R protein was purified as described in example 1. In example 2, amino acid sequence information derived from fragments of TRAIL-R is presented. One embodiment of the invention is directed to a purified human TRAIL-R protein that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD (amino acids 327 to 333 of SEQ ID NO:2). In another embodiment, the TRAIL-R additionally comprises the sequence ETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLXTML (amino acids 336 to 386 of SEQ ID NO:2, with one unknown amino acid indicated as X). Also provided are TRAIL-R fragments comprising only one of these characterizing amino acid sequences.

The nucleotide sequence of a TRAIL-R DNA fragment, and the amino acid sequence encoded thereby, are presented in FIG. **1** (SEQ ID NO:3 and SEQ ID NO:4); see example 3. The amino acid sequence presented in FIG. **1** has characteristics of the so-called "death domains" found in the cytoplasmic region of certain other receptor proteins. Such domains have been reported to be associated with transduction of apoptotic signals. Cytoplasmic death domains have been identified in Fas antigen (Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993), TNF receptor type I (Tartaglia et al. *Cell* 74:845, 1993), DR3 (Chinnaiyan et al., *Science* 274:990–992, 1996), and CAR-1 (Brojatsch et al., *Cell* 87:845–855, 1996). The role of these death domains in initiating intracellular apoptotic signaling cascades is discussed further below.

SEQ ID NO:1 presents the nucleotide sequence of the coding region of a human TRAIL receptor DNA, including an initiation codon (ATG) and a termination codon (TAA). The amino acid sequence encoded by the DNA of SEQ ID NO:1 is presented in SEQ ID NO:2. The fragment depicted in FIG. **1** corresponds to the region of TRAIL-R that is presented as amino acids 336 to 386 in SEQ ID NO:2.

The TRAIL-R protein of SEQ ID NO:2 includes an N-terminal hydrophobic region that functions as a signal peptide, followed by an extracellular domain, a transmembrane region comprising amino acids 211 through 231, and a C-terminal cytoplasmic domain. Computer analysis predicts that the signal peptide corresponds to residues 1 to 51 of SEQ ID NO:2. Cleavage of the signal peptide thus would yield a mature protein comprising amino acids 52 through 440 of SEQ ID NO:2. The calculated molecular weight for a mature protein containing residues 52 to 440 of SEQ ID NO:2 is about 43 kilodaltons. The next most likely computer-predicted signal peptidase cleavage sites (in descending order) occur after amino acids 50 and 58 of SEQ ID NO:2.

In another embodiment of the invention, the N-terminal residue of a mature TRAIL-R protein is the isoleucine residue at position 56 of SEQ ID NO:2. Sequences of several tryptic digest peptide fragments of TRAIL-R were determined by a combination of N-terminal sequencing and Nano-ES MS/MS (nano electrospray tandem mass spectrometry). The N-terminal amino acid of one of the peptide fragments was the isoleucine at position 56 of SEQ ID NO:2. Since this fragment was not preceded by a trypsin cleavage site, the (Ile)56 residue may correspond to the N-terminal residue resulting from cleavage of the signal peptide.

A further embodiment of the invention is directed to mature TRAIL-R having amino acid 54 as the N-terminal residue. In one preparation of TRAIL-R (a soluble TRAIL-R/Fc fusion protein expressed in CV1-EBNA cells), the signal peptide was cleaved after residue 53 of SEQ ID NO:2.

The skilled artisan will recognize that the molecular weight of particular preparations of TRAIL-R protein may differ, according to such factors as the degree of glycosylation. The glycosylation pattern of a particular preparation of TRAIL-R may vary according to the type of cells in which the protein is expressed, for example. Further, a given preparation may include multiple differentially glycosylated species of the protein. TRAIL-R polypeptides with or without associated native-pattern glycosylation are provided herein. Expression of TRAIL-R polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

In one embodiment, the protein is characterized by a molecular weight within the range of about 50 to 55 kilodaltons, which is the molecular weight determined for a preparation of native, full length, human TRAIL-R. Molecular weight can be determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Example 1 presents one method for purifying a TRAIL-R protein. Jurkat cells are disrupted, and the subsequent purification process includes affinity chromatography (employing a chromatography matrix containing TRAIL), and reversed phase HPLC.

TRAIL-R polypeptides of the present invention may be purified by any suitable alternative procedure, using known protein purification techniques. In one alternative procedure, the chromatography matrix instead comprises an antibody that binds TRAIL-R. Other cell types expressing TRAIL-R (e.g., the PS-1 cells described in example 2) can be substituted for the Jurkat cells. The cells can be disrupted by any of the numerous known techniques, including freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered in vivo, for example. Advantageously, TRAIL-R polypeptides are purified such that no protein bands corresponding to other (non-TRAIL-R) proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to TRAIL-R protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. TRAIL-R most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

The present invention encompasses TRAIL-R in various forms, including those that are naturally occurring or produced through various techniques such as procedures involving recombinant DNA technology. Such forms of TRAIL-R include, but are not limited to, fragments, derivatives, variants, and oligomers of TRAIL-R, as well as fusion proteins containing TRAIL-R or fragments thereof.

TRAIL-R may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL-R may be prepared by linking the chemical moieties to functional groups on TRAIL-R amino acid side chains or at the N-terminus or C-terminus of a TRAIL-R polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached to TRAIL-R are contemplated herein, as discussed in more detail below.

Other derivatives of TRAIL-R within the scope of this invention include covalent or aggregative conjugates of TRAIL-R polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with TRAIL-R oligomers. Further, TRAIL-R-containing fusion proteins can comprise peptides added to facilitate purification and identification of TRAIL-R. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Pat. No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the Flag® peptide in the presence of certain divalent metal cations, as described in U.S. Pat. No. 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Conn.

Both cell membrane-bound and soluble (secreted) forms of TRAIL-R are provided herein. Soluble TRAIL-R may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells expressing a TRAIL-R polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL-R in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

Soluble forms of receptor proteins typically lack the transmembrane region that would cause retention of the protein on the cell surface. In one embodiment of the invention, a soluble TRAIL-R polypeptide comprises the extracellular domain of the protein. A soluble TRAIL-R polypeptide may include the cytoplasmic domain, or a portion thereof, as long as the polypeptide is secreted from the cell in which it is produced. One example of a soluble TRAIL-R is a soluble human TRAIL-R comprising amino acids 52 to 210 of SEQ ID NO:2. Other soluble TRAIL-R polypeptides include, but are not limited to, polypeptides comprising amino acids x to 210 of SEQ ID NO:2, wherein x is an integer from 51 through 59.

Soluble forms of TRAIL-R possess certain advantages over the membrane-bound form of the protein. Purification

of the protein from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for certain applications, e.g., for intravenous administration.

TRAIL-R fragments are provided herein. Such fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative involves generating TRAIL-R fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed as the 5' and 3' primers in the PCR.

Examples of fragments are those comprising at least 20, or at least 30, contiguous amino acids of the sequence of SEQ ID NO:2. Fragments derived from the cytoplasmic domain find use in studies of TRAIL-R-mediated signal transduction, and in regulating cellular processes associated with transduction of biological signals. TRAIL-R polypeptide fragments also may be employed as immunogens, in generating antibodies. Particular embodiments are directed to TRAIL-R polypeptide fragments that retain the ability to bind TRAIL. Such a fragment may be a soluble TRAIL-R polypeptide, as described above.

Naturally occurring variants of the TRAIL-R protein of SEQ ID NO:2 are provided herein. Such variants include, for example, proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the TRAIL-R protein. Alternate splicing of mRNA may, for example, yield a truncated but biologically active TRAIL-R protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL-R protein (generally from 1–5 terminal amino acids). TRAIL-R proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

The skilled artisan will also recognize that the position(s) at which the signal peptide is cleaved may differ from that predicted by computer program, and may vary according to such factors as the type of host cells employed in expressing a recombinant TRAIL-R polypeptide. A protein preparation may include a mixture of protein molecules having different N-terminal amino acids, resulting from cleavage of the signal peptide at more than one site. As discussed above, particular embodiments of mature TRAIL-R proteins provided herein include, but are not limited to, proteins having the residue at position 51, 52, 54, 56, or 59 of SEQ ID NO:2 as the N-terminal amino acid.

Regarding the discussion herein of various domains of TRAIL-R protein, the skilled artisan will recognize that the above-described boundaries of such regions of the protein are approximate. To illustrate, the boundaries of the transmembrane region (which may be predicted by using computer programs available for that purpose) may differ from those described above. Thus, soluble TRAIL-R polypeptides in which the C-terminus of the extracellular domain differs from the residue so identified above are contemplated herein.

Other naturally occurring TRAIL-R DNAs and polypeptides include those derived from non-human species. Homologs of the human TRAIL-R of SEQ ID NO:2, from other mammalian species, are contemplated herein, for example. Probes based on the human DNA sequence of SEQ ID NO:3 or SEQ ID NO:1 may be used to screen cDNA libraries derived from other mammalian species, using conventional cross-species hybridization techniques.

TRAIL-R DNA sequences may vary from the native sequences disclosed herein. Due to the known degeneracy of the genetic code, wherein more than one codon can encode the same amino acid, a DNA sequence can vary from that shown in SEQ ID NO:1 and still encode a TRAIL-R protein having the amino acid sequence of SEQ ID NO:2. Such variant DNA sequences may result from silent mutations (e.g., occurring during PCR amplification), or may be the product of deliberate mutagenesis of a native sequence. Thus, among the DNA sequences provided herein are native TRAIL-R sequences (e.g., cDNA comprising the nucleotide sequence presented in SEQ ID NO:1) and DNA that is degenerate as a result of the genetic code to a native TRAIL-R DNA sequence.

Among the TRAIL-R polypeptides provided herein are variants of native TRAIL-R polypeptides that retain a biological activity of a native TRAIL-R. Such variants include polypeptides that are substantially homologous to native TRAIL-R, but which have an amino acid sequence different from that of a native TRAIL-R because of one or more deletions, insertions or substitutions. Particular embodiments include, but are not limited to, TRAIL-R polypeptides that comprise from one to ten deletions, insertions or substitutions of amino acid residues, when compared to a native TRAIL-R sequence. The TRAIL-R-encoding DNAs of the present invention include variants that differ from a native TRAIL-R DNA sequence because of one or more deletions, insertions or substitutions, but that encode a biologically active TRAIL-R polypeptide. One biological activity of TRAIL-R is the ability to bind TRAIL.

Nucleic acid molecules capable of hybridizing to the DNA of SEQ ID NO:1 or SEQ ID NO:3 under moderately stringent or highly stringent conditions, and which encode a biologically active TRAIL-R, are provided herein. Such hybridizing nucleic acids include, but are not limited to, variant DNA sequences and DNA derived from non-human species, e.g., non-human mammals.

Moderately stringent conditions include conditions described in, for example, Sambrook et al, *Molecular Cloning: A Laboratory Manual,* 2nd ed., Vol. 1, pp 1.101–104, Cold Spring Harbor Laboratory Press, 1989. Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5×SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55° C., 5×SSC, overnight. Highly stringent conditions include higher temperatures of hybridization and washing. One embodiment of the invention is directed to DNA sequences that will hybridize to the DNA of SEQ ID NOS:1 or 3 under highly stringent conditions, wherein said conditions include hybridization at 68° C. followed by washing in 0.1×SSC/0.1% SDS at 63–68° C.

Certain DNAs and polypeptides provided herein comprise nucleotide or amino acid sequences, respectively, that are at least 80% identical to a native TRAIL-R sequence. Also contemplated are embodiments in which a TRAIL-R DNA or polypeptide comprises a sequence that is at least 90% identical, at least 95% identical, or at least 98% identical to a native TRAIL-R sequence. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described

by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353–358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

In particular embodiments of the invention, a variant TRAIL-R polypeptide differs in amino acid sequence from a native TRAIL-R, but is substantially equivalent to a native TRAIL-R in a biological activity. One example is a variant TRAIL-R that binds TRAIL with essentially the same binding affinity as does a native TRAIL-R. Binding affinity can be measured by conventional procedures, e.g., as described in U.S. Pat. No. 5,512,457.

Variant amino acid sequences may comprise conservative substitution(s), meaning that one or more amino acid residues of a native TRAIL-R is replaced by a different residue, but that the conservatively substituted TRAIL-R polypeptide retains a desired biological activity of the native protein (e.g., the ability to bind TRAIL). A given amino acid may be replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other conservative substitutions, e.g., involving substitutions of entire regions having similar hydrophobicity characteristics, are well known.

In another example of variants, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Certain receptors of the TNF-R family contain cysteine-rich repeat motifs in their extracellular domains (Marsters et al., *J. Biol. Chem.* 267:5747–5750, 1992). These repeats are believed to be important for ligand binding. To illustrate, Marsters et al., supra, reported that soluble TNF-R type 1 polypeptides lacking one of the repeats exhibited a ten fold reduction in binding affinity for TNFα and TNFβ; deletion of the second repeat resulted in a complete loss of detectable binding of the ligands. The human TRAIL-R of SEQ ID NO:2 contains two such cysteine rich repeats, the first including residues 94 through 137, and the second including residues 138 through 178. Cysteine residues within these cysteine rich domains advantageously remain unaltered in TRAIL-R variants, when retention of TRAIL-binding activity is desired.

Other variants are prepared by modification of adjacent dibasic amino acid residues, to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Mature human TRAIL-R contains such adjacent basic residue pairs at amino acids 72–73, 154–155, 322–323, 323–324, and 359–360 of SEQ ID NO:2. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

TRAIL-R polypeptides, including variants and fragments thereof, can be tested for biological activity in any suitable assay. The ability of a TRAIL-R polypeptide to bind TRAIL can be confirmed in conventional binding assays, examples of which are described below.

Expression Systems

The present invention also provides recombinant cloning and expression vectors containing TRAIL-R DNA, as well as host cell containing the recombinant vectors. Expression vectors comprising TRAIL-R DNA may be used to prepare TRAIL-R polypeptides encoded by the DNA. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. The skilled artisan will recognize that the procedure for purifying the expressed TRAIL-R will vary according to such factors as the type of host cells employed, and whether the TRAIL-R is membrane-bound or a soluble form that is secreted from the host cell.

Any suitable expression system may be employed. The vectors include a DNA encoding a TRAIL-R polypeptide, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the TRAIL-R DNA sequence. Thus, a promoter nucleotide sequence is operably linked to an TRAIL-R DNA sequence if the promoter nucleotide sequence controls the transcription of the TRAIL-R DNA sequence. An origin of replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

In addition, a sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in frame to the TRAIL-R sequence so that the TRAIL-R is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the TRAIL-R polypeptide. The signal peptide is cleaved from the TRAIL-R polypeptide upon secretion of TRAIL-R from the cell.

Suitable host cells for expression of TRAIL-R polypeptides include prokaryotes, yeast or higher eukaryotic cells. Mammalian or insect cells are generally preferred for use as host cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y., (1985). Cell-free translation systems could also be employed to produce TRAIL-R polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or Bacilli. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium*, and various other species within the genera Pseudomonas, Streptomyces, and Staphylococcus. In a prokaryotic host cell, such as *E. coli*, a TRAIL-R polypeptide may include an N-terminal methionine residue to facilitate expression of the

recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant TRAIL-R polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a TRAIL-R DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, Wis., USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RR1, ATCC 53082).

TRAIL-R alternatively may be expressed in yeast host cells, preferably from the Saccharomyces genus (e.g., *S. cerevisiae*). Other genera of yeast, such as Pichia or Kluyveromyces, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the TRAIL polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp$^+$ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems also may be employed to express recombinant TRAIL-R polypeptides. Bacculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV1/EBNA cell line derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the SV40 viral origin of replication site is included.

Expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983), for example. A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature* 312:768, 1984 has been deposited as ATCC 39890. Additional mammalian expression vectors are described in EP-A-0367566, and in WO 91/18982. As one alternative, the vector may be derived from a retrovirus.

Overexpression of full length TRAIL-R has resulted in membrane blebbing and nuclear condensation of transfected CV-1/EBNA cells, indicating that the mechanism of cell death was apoptosis. For host cells in which such TRAIL-R-mediated apoptosis occurs, a suitable apoptosis inhibitor may be included in the expression system.

To inhibit TRAIL-R-induced apoptosis of host cells expressing recombinant TRAIL-R, the cells may be

co-transfected with an expression vector encoding a polypeptide that functions as an apoptosis inhibitor. Expression vectors encoding such polypeptides can be prepared by conventional procedures. Another approach involves adding an apoptosis inhibitor to the culture medium. The use of poxvirus CrmA, baculovirus P35, a C-terminal fragment of FADD, and the tripeptide derivative zVAD-fmk, to reduce death of host cells is illustrated in examples 6 and 8.

zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) is a tripeptide based compound, available from Enzyme System Products, Dublin, Calif. As illustrated in example 8, zVAD-fmk may be added to the medium in which cells expressing TRAIL-R are cultured.

The 38-kilodalton cowpox-derived protein that was subsequently designated CrmA is described in Pickup et al. (*Proc. Natl. Acad. Sci. USA* 83:7698–7702, 1986; hereby incorporated by reference). Sequence information for CrmA is presented in FIG. 4 of Pickup et al., supra. One approach to producing and purifying CrmA protein is described in Ray et al. (*Cell,* 69:597–604, 1992; hereby incorporated by reference).

A 35-kilodalton protein encoded by *Autographa californica* nuclear polyhedrosis virus, a baculovirus, is described in Friesen and Miller (*J. Virol.* 61:2264–2272, 1987; hereby incorporated by reference). Sequence information for this protein, designated baculovirus p35 herein, is presented in FIG. 5 of Friesen and Miller, supra.

The death domain-containing cytoplasmic protein FADD (also known as MORT1) is described in Boldin et al. (*J. Biol. Chem.* 270:7795–7798, 1995; hereby incorporated by reference). FADD has been reported to associate, directly or indirectly, with the cytoplasmic death domain of certain receptors that mediate apoptosis (Boldin et al., *Cell* 85:803–815, June 1996; Hsu et al., *Cell* 84:299–308, January 1996).

In one embodiment of the present invention, truncated FADD polypeptides that include the death domain (located in the C-terminal portion of the protein), but lack the N-terminal region to which apoptosis effector functions have been attributed, are employed to reduce apoptosis. The use of certain FADD deletion mutant polypeptides, truncated at the N-terminus, to inhibit death of cells expressing other apoptosis-inducing receptors, is described in Hsu et al. (*Cell* 84:299–308, 1996; hereby incorporated by reference).

This approach is illustrated in example 8, which employs one suitable FADD-dominant negative (FADD-DN) polypeptide, having an amino acid sequence corresponding to amino acids 117 through 245 of the MORT1 amino acid sequence presented in Boldin et al. (*J. Biol. Chem.* 270:7795–7798, 1995). In example 8, cells were co-transfected with a TRAIL-R-encoding expression vector, and with an expression vector encoding the above-described Flag® peptide, fused to the N-terminus of the FADD-DN polypeptide.

While not wishing to be bound by theory, one possible explanation is that the C-terminal fragments of FADD associate with the intracellular death domain of the receptor, but lack the N-terminal portion of the protein that is necessary for effecting apoptosis (Hsu et al., *Cell* 84:299–308, January 1996; Boldin et al., *Cell* 85:803–815, June 1996). The truncated FADD thereby may block association of endogenous, full length FADD with the receptor's death domain; consequently, the apoptosis that would be initiated by such endogenous FADD is inhibited.

Other apoptosis inhibitors useful in expression systems of the present invention can be identified in conventional assay procedures. One such assay, in which compounds are tested for the ability to reduce apoptosis of cells expressing TRAIL-R, is described in example 8.

Poxvirus CrmA, baculovirus P35, and zVAD-fmk are viral caspase inhibitors. Other caspase inhibitors may be tested for the ability to reduce TRAIL-R-mediated cell death.

The use of CrmA, baculovirus p35, and certain peptide derivatives (including zVAD-fmk) as inhibitors of apoptosis in particular cells/systems is discussed in Sarin et al. (*J. Exp. Med.* 184:2445–2450, December 1996; hereby incorporated by reference). The role of interleukin-1β converting enzyme (ICE) family proteases in signal transduction cascades leading to programmed cell death, and the use of inhibitors of such proteases to block apoptosis, is discussed in Sarin et al., supra, and Muzio et al., *Cell* 85:817–827, 1996).

Apoptosis inhibitors generally need not be employed for expression of TRAIL-R polypeptides lacking the cytoplasmic domain (i.e., lacking the region of the protein involved in signal transduction). Thus, expression systems for producing soluble TRAIL-R polypeptides comprising only the extracellular domain (or a fragment thereof) need not include one of the above-described apoptosis inhibitors.

Regarding signal peptides that may be employed in producing TRAIL-R, the native signal peptide of TRAIL-R may be replaced by a heterologous signal peptide or leader sequence, if desired. The choice of signal peptide or leader may depend on factors such as the type of host cells in which the recombinant TRAIL-R is to be produced. To illustrate, examples of heterologous signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in U.S. Pat. 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Pat. 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

Oligomeric Forms of TRAIL-R

Encompassed by the present invention are oligomers that contain TRAIL-R polypeptides. TRAIL-R oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers.

One embodiment of the invention is directed to oligomers comprising multiple TRAIL-R polypeptides joined via covalent or non-covalent interactions between peptide moieties fused to the TRAIL-R polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of TRAIL-R polypeptides attached thereto, as described in more detail below.

In particular embodiments, the oligomers comprise from two to four TRAIL-R polypeptides. The TRAIL-R moieties of the oligomer may be soluble polypeptides, as described above.

As one alternative, a TRAIL-R oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1–10.19.11, 1992).

One embodiment of the present invention is directed to a TRAIL-R dimer comprising two fusion proteins created by

fusing TRAIL-R to the Fc region of an antibody. A gene fusion encoding the TRAIL-R/Fc fusion protein is inserted into an appropriate expression vector. TRAIL-R/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent TRAIL-R.

Provided herein are fusion proteins comprising a TRAIL-R polypeptide fused to an Fc polypeptide derived from an antibody. DNA encoding such fusion proteins, as well as dimers containing two fusion proteins joined via disulfide bonds between the Fc moieties thereof, are also provided. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included.

One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Pat. No. 5,457,035 and in Baum et al., (*EMBO J.* 13:3992–4001, 1994). The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

In other embodiments, TRAIL-R may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a TRAIL-R oligomer with as many as four TRAIL-R extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple TRAIL-R polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Pat. Nos. 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TRAIL-R, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between sequences encoding TRAIL-R. In particular embodiments, a fusion protein comprises from two to four soluble TRAIL-R polypeptides, separated by peptide linkers.

Another method for preparing oligomeric TRAIL-R involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize.

Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, and the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994), hereby incorporated by reference. The use of a modified leucine zipper that allows for stable trimerization of a heterologous protein fused thereto is described in Fanslow et al. (*Semin. Immunol.* 6:267–278, 1994). Recombinant fusion proteins comprising a soluble TRAIL-R polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble oligomeric TRAIL-R that forms is recovered from the culture supernatant.

Oligomeric TRAIL-R has the property of bivalent, trivalent, etc. binding sites for TRAIL. The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns. DNA sequences encoding oligomeric TRAIL-R, or encoding fusion proteins useful in preparing TRAIL-R oligomers, are provided herein.

Assays

TRAIL-R proteins (including fragments, variants, oligomers, and other forms of TRAIL-R) may be tested for the ability to bind TRAIL in any suitable assay, such as a conventional binding assay. To illustrate, TRAIL-R may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled TRAIL-R is contacted with cells expressing TRAIL. The cells then are washed to remove unbound labeled TRAIL-R, and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing TRAIL cDNA is constructed, e.g., as described in in PCT application WO 97/01633, hereby incorporated by reference. DNA and amino acid sequence information for human and mouse TRAIL is presented in WO 97/01633. TRAIL comprises an N-terminal cytoplasmic domain, a transmembrane region, and a C-terminal extracellular domain. CV1-EBNA-1 cells in 10 cm^2 dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about 4×10^4 cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37° C. with various concentrations of a soluble TRAIL-R/Fc fusion protein. Cells then are washed and incubated with a constant saturating concentration of a ^{125}I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37° C. After extensive washing, cells are released via trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, Pa. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any TRAIL-R/Fc protein that has bound to the cells. In all assays, non-specific binding of ^{125}I-antibody is assayed in the absence of TRAIL-R/Fc, as well as in the presence of TRAIL-R/Fc and a 200-fold molar excess of unlabeled mouse anti-human IgG antibody.

Cell-bound ^{125}I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, Mass.) run on a Microvax computer.

Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a TRAIL-R variant may be determined by assaying for the variant's ability to compete with a native TRAIL-R for binding to TRAIL.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled TRAIL-R and intact cells expressing TRAIL (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble TRAIL-R fragment can be used to compete with a soluble TRAIL-R variant for binding to cell surface TRAIL. Instead of intact cells, one could substitute a soluble TRAIL/Fc fusion protein bound to a solid phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, N.J. Another type of competitive binding assay utilizes radiolabeled soluble TRAIL, such as a soluble TRAIL/Fc fusion protein, and intact cells expressing TRAIL-R. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

Another type of assay for biological activity involves testing a TRAIL-R polypeptide for the ability to block TRAIL-mediated apoptosis of target cells, such as the human leukemic T-cell line known as Jurkat cells, for example. TRAIL-mediated apoptosis of the cell line designated Jurkat clone E6-1 (ATCC TIB 152) is demonstrated in assay procedures described in PCT application WO 97/01633, hereby incorporated by reference.

Uses of TRAIL-R

Uses of TRAIL-R include, but are not limited to, the following. Certain of these uses of TRAIL-R flow from its ability to bind TRAIL.

TRAIL-R finds use as a protein purification reagent. TRAIL-R polypeptides may be attached to a solid support material and used to purify TRAIL proteins by affinity chromatography. In particular embodiments, a TRAIL-R polypeptide (in any form described herein that is capable of binding TRAIL) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, N.J.). In an alternative, a TRAIL-R/Fc protein is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

TRAIL-R proteins also find use in measuring the biological activity of TRAIL proteins in terms of their binding affinity for TRAIL-R. TRAIL-R proteins thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of TRAIL protein under different conditions. To illustrate, TRAIL-R may be employed in a binding affinity study to measure the biological activity of a TRAIL protein that has been stored at different temperatures, or produced in different cell types. TRAIL-R also may be used to determine whether biological activity is retained after modification of a TRAIL protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified TRAIL protein for TRAIL-R is compared to that of an unmodified TRAIL protein to detect any adverse impact of the modifications on biological activity of TRAIL. The biological activity of a TRAIL protein thus can be ascertained before it is used in a research study, for example.

TRAIL-R also finds use in purifying or identifying cells that express TRAIL on the cell surface. TRAIL-R polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with TRAIL-R and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing TRAIL-expressing cells are contacted with the solid phase having TRAIL-R thereon. Cells expressing TRAIL on the cell surface bind to the fixed TRAIL-R, and unbound cells then are washed away.

Alternatively, TRAIL-R can be conjugated to a detectable moiety, then incubated with cells to be tested for TRAIL expression. After incubation, unbound labeled TRAIL-R is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing $TRAIL^+$ cells are incubated with biotinylated TRAIL-R. Incubation periods are typically at least one hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.,* 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

TRAIL-R polypeptides also find use as carriers for delivering agents attached thereto to cells bearing TRAIL. Cells expressing TRAIL include those identified in Wiley et al. (*Immunity,* 3:673–682, 1995). TRAIL-R proteins thus can be used to deliver diagnostic or therapeutic agents to such cells (or to other cell types found to express TRAIL on the cell surface) in in vitro or in vivo procedures.

Detectable (diagnostic) and therapeutic agents that may be attached to a TRAIL-R polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a calorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to, ^{123}I, ^{131}I, ^{99m}Tc, ^{111}In, and ^{76}Br. Examples of radionuclides suitable for therapeutic use are ^{131}I, ^{211}At, ^{77}Br, ^{186}Re, ^{188}Re, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, and ^{67}Cu.

Such agents may be attached to the TRAIL-R by any suitable conventional procedure. TRAIL-R, being a protein, comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Ill.) A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to TRAIL-R by using a suitable bifunctional chelating agent, for example.

Conjugates comprising TRIAL-R and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

TRIAL-R DNA and polypeptides of the present invention may be used in developing treatments for any disorder

mediated (directly or indirectly) by defective, or insufficient amounts of, TRIAL-R. TRIAL-R polypeptides may be administered to a mammal afflicted with such a disorder. Alternatively, a gene therapy approach may be taken. Disclosure herein of native TRAIL-R nucleotide sequences permits the detection of defection TRIAL-R genes, and the replacement thereof with normal TRIAL-R-encoding genes. Defective genes mat be detected in in vitro diagnostic assays, and by comparision of a native TRAIL-R nucleotide sequence disclosed herein with that of a TRAIL-R gene derived from a person suspected of harboring a defect in this gene.

Another use of the protein of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL-R interactions on different cell types. TRAIL-R polypeptides also may be employed in in vitro assays for detecting TRAIL or TRAIL-R or the interactions thereof.

TRAIL-R may be employed in inhibiting a biological activity of TRAIL, in in vitro or in vivo procedures. A purified TRAIL-R polypeptide may be used to inhibit binding of TRAIL to endogenous cell surface TRAIL-R. Biological effects that result from the binding of TRAIL to endogenous receptors thus are inhibited. Various forms of TRAIL-R may be employed, including, for example, the above-described TRAIL-R fragments, oligomers, derivatives, and variants that are capable of binding TRAIL. In one embodiment, a soluble TRAIL-R is employed to inhibit a biological activity of TRAIL, e.g., to inhibit TRAIL-mediated apoptosis of particular cells.

TRAIL-R may be administered to a mammal to treat a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

TRAIL-R may be useful for treating thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H. C., *Semin. Hematol.,* 24:71, 1987; Thompson et al., *Blood,* 80:1890, 1992). Increasing TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok et al., *Am. J. Hematol.* 50:84, 1995).

Plasma from patients afflicted with TTP (including HIV^+ and HIV^- patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence et al., *Blood,* 87:3245, Apr. 15, 1996). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in PCT application WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and may play a role in inducing apoptosis of microvascular endothelial cells.

Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J. L., *Lancet,* 343:393, 1994; Melnyk et al., (*Arch. Intern. Med.,* 155:2077, 1995; Thompson et al., supra). One embodiment of the invention is directed to use of TRAIL-R to treat the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS.

Other conditions characterized by clotting of small blood vessels may be treated using TRAIL-R. Such conditions include but are not limited to the following. Cardiac problems seen in about 5–10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has been reported in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R ex vivo. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized receptor binds TRAIL, thus removing TRAIL protein from the patient's blood.

Alternatively, TRAIL-R may be administered in vivo to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of TRAIL-R is administered to the patient.

The present invention thus provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of TRAIL-R. A TRAIL-R polypeptide may be employed in in vivo or ex vivo procedures, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TRAIL-R may be employed in conjunction with other agents useful in treating a particular disorder. In an in vitro study reported by Laurence et al. (*Blood* 87:325, 1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate.

Thus, a patient may be treated with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, in combination with an agent that inhibits TRAIL-mediated apoptosis of endothelial cells. In one embodiment, TRAIL-R and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in PCT application publication number WO 95/10540, hereby incorporated by reference.

Another embodiment of the present invention is directed to the use of TRAIL-R to reduce TRAIL-mediated death of T cells in HIV-infected patients. The role of T cell apoptosis in the development of AIDS has been the subject of a number of studies (see, for example, Meyaard et al., *Science* 257:217–219, 1992; Groux et al., *J Exp. Med.,* 175:331, 1992; and Oyaizu et al., in *Cell Activation and Apoptosis in HIV Infection*, Andrieu and Lu, Eds., Plenum Press, New York, 1995, pp. 101–114). Certain investigators have studied the role of Fas-mediated apoptosis; the involvement of interleukin-1β-converting enzyme (ICE) also has been explored (Estaquier et al., *Blood* 87:4959–4966, 1996; Mitra et al., *Immunology* 87:581–585, 1996; Katsikis et al., *J. Exp. Med.* 181:2029–2036, 1995). It is possible that T cell apoptosis occurs through multiple mechanisms.

At least some of the T cell death seen in HIV^+ patients is believed to be mediated by TRAIL. While not wishing to be bound by theory, such TRAIL-mediated T cell death is believed to occur through the mechanism known as activation-induced cell death (AICD).

Activated human T cells are induced to undergo programmed cell death (apoptosis) upon triggering through the CD3/T cell receptor complex, a process termed activated-induced cell death (AICD). AICD of $CD4^+$ T cells isolated from HIV-infected aymptomatic individuals has been reported (Groux et al., supra). Thus, AICD may play a role in the depletion of $CD4^+$ T cells and the progression to AIDS in HIV-infected individuals.

The present invention provides a method of inhibiting TRAIL-mediated T cell death in HIV^+ patients, comprising administering TRAIL-R (preferably, a soluble TRAIL-R polypeptide) to the patients. In one embodiment, the patient

is asymptomatic when treatment with TRAIL-R commences. If desired, prior to treatment, peripheral blood T cells may be extracted from an HIV+ patient, and tested for susceptibility to TRAIL-mediated cell death by conventional procedures.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R ex vivo. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized TRAIL-R binds TRAIL, thus removing TRAIL protein from the patient's blood.

In treating HIV+ patients, TRAIL-R may be employed in combination with other inhibitors of T cell apoptosis. Fas-mediated apoptosis also has been implicated in loss of T cells in HIV+ individuals (Katsikis et al., *J. Exp. Med.* 181:2029–2036, 1995). Thus, a patient susceptible to both Fas ligand (Fas-L)-mediated and TRAIL-mediated T cell death may be treated with both an agent that blocks TRAIL/TRAIL-R interactions and an agent that blocks Fas-L/Fas interactions. Suitable agents for blocking binding of Fas-L to Fas include, but are not limited to, soluble Fas polypeptides; oligomeric forms of soluble Fas polypeptides (e.g., dimers of sFas/Fc); anti-Fas antibodies that bind Fas without transducing the biological signal that results in apoptosis; anti-Fas-L antibodies that block binding of Fas-L to Fas; and muteins of Fas-L that bind Fas but don't transduce the biological signal that results in apoptosis. Preferably, the antibodies employed in the method are monoclonal antibodies. Examples of suitable agents for blocking Fas-L/Fas interactions, including blocking anti-Fas monoclonal antibodies, are described in WO 95/10540, hereby incorporated by reference.

Compositions comprising an effective amount of a TRAIL-R polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL-R can be formulated according to known methods used to prepare pharmaceutically useful compositions. TRAIL-R can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences,* 16th ed. 1980, Mack Publishing Company, Easton, Pa.

In addition, such compositions can contain TRAIL-R complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance of TRAIL-R, and are thus chosen according to the intended application. TRAIL-R expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL-R polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition comprises a soluble TRAIL-R polypeptide or an oligomer comprising soluble TRAIL-R polypeptides.

TRAIL-R can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration.

Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

Compositions comprising TRAIL-R nucleic acids in physiologically acceptable formulations are also contemplated. TRAIL-R DNA may be formulated for injection, for example.

Antibodies

Antibodies that are immunoreactive with TRAIL-R polypeptides are provided herein. Such antibodies specifically bind TRAIL-R, in that the antibodies bind to TRAIL-R via the antigen-binding sites of the antibody (as opposed to non-specific binding).

The TRAIL-R protein prepared as described in example 1 may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively, another form of TRAIL-R, such as a fragment or fusion protein, is employed as the immunogen.

Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1988). Production of monoclonal antibodies directed against TRAIL-R is further illustrated in example 4.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature* 332:323, 1988), Liu et al. (*PNAS* 84:3439, 1987), Larrick et al. (*Bio/Technology* 7:934, 1989), and Winter and Harris (*TIPS* 14:139, May, 1993).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL-R polypeptides, either in vitro or in vivo. The antibodies also may be employed in purifying TRAIL-R proteins by immunoaffinity chromatography.

Those antibodies that additionally can block binding of TRAIL-R to TRAIL may be used to inhibit a biological

activity that results from such binding. Such blocking antibodies may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of TRAIL to cells expressing TRAIL-R. Examples of such cells are the Jurkat cells and PS 1 cells described in example 2 below. Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of TRAIL to target cells. Antibodies may be assayed for the ability to inhibit TRAIL-mediated lysis of Jurkat cells, for example.

Such an antibody may be employed in an in vitro procedure, or administered in vivo to inhibit a TRAIL-R-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL with cell surface TRAIL receptor thus may be treated. A therapeutic method involves in vivo administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-mediated biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

A blocking antibody directed against TRAIL-R may be substituted for TRAIL-R in the above-described method of treating thrombotic microangiopathy, e.g., in treating TTP or HUS. The antibody is administered in vivo, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

Antibodies raised against TRAIL-R may be screened for agonistic (i.e., ligand-mimicking) properties. Such antibodies, upon binding to cell surface TRAIL-R, induce biological effects (e.g., transduction of biological signals) similar to the biological effects induced when TRAIL binds to cell surface TRAIL-R. Agonistic antibodies may be used to induce apoptosis of certain cancer cells or virally infected cells, as has been reported for TRAIL. The ability of TRAIL to kill cancer cells (including but not limited to leukemia, lymphoma, and melanoma cells) and virally infected cells is described in Wiley et al. (*Immunity* 3:673–682, 1995); and in PCT application WO 97/01633.

Compositions comprising an antibody that is directed against TRAIL-R, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing TRAIL-R proteins.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL-R. Examples of such agents are presented above. The conjugates find use in in vitro or in vivo procedures.

Nucleic Acids

The present invention provides TRAIL-R nucleic acids. Such nucleic acids include, but are not limited to, DNA encoding the peptide described in example 2. Such DNAs can be identified from knowledge of the genetic code. Other nucleic acids of the present invention include isolated DNAs comprising the nucleotide sequence presented in SEQ ID NO:1 or SEQ ID NO:3.

The present invention provides isolated nucleic acids useful in the production of TRAIL-R polypeptides, e.g., in the recombinant expression systems discussed above. Such nucleic acids include, but are not limited to, the human TRAIL-R DNA of SEQ ID NO:1. Nucleic acid molecules of the present invention include TRAIL-R DNA in both single-stranded and double-stranded form, as well as the RNA complement thereof. TRAIL-R DNA includes, for example, cDNA, genomic DNA, chemically synthesized DNA, DNA amplified by PCR, and combinations thereof. Genomic DNA may be isolated by conventional techniques, e.g., using the cDNA of SEQ ID NO:1 or 3, or a suitable fragment thereof, as a probe.

DNAs encoding TRAIL-R in any of the forms contemplated herein (e.g., full length TRAIL-R or fragments thereof) are provided. Particular embodiments of TRAIL-R-encoding DNAs include a DNA encoding the full length human TRAIL-R of SEQ ID NO:2 (including the N-terminal signal peptide), and a DNA encoding a full length mature human TRAIL-R. Other embodiments include DNA encoding a soluble TRAIL-R (e.g., encoding the extracellular domain of the protein of SEQ ID NO:2, either with or without the signal peptide).

One embodiment of the invention is directed to fragments of TRAIL-R nucleotide sequences comprising at least about 17 contiguous nucleotides of a TRAIL-R DNA sequence. In other embodiments, a DNA fragment comprises at least 30, or at least 60, contiguous nucleotides of a TRAIL-R DNA sequence. Nucleic acids provided herein include DNA and RNA complements of said fragments, along with both single-stranded and double-stranded forms of the TRAIL-R DNA.

Among the uses of TRAIL-R nucleic acid fragments is use as probes or primers. Using knowledge of the genetic code in combination with the amino acid sequences set forth in example 2, sets of degenerate oligonucleotides can be prepared. Such oligonucleotides find use as primers, e.g., in polymerase chain reactions (PCR), whereby TRAIL-R DNA fragments are isolated and amplified.

Other useful fragments of the TRAIL-R nucleic acids include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target TRAIL-R mRNA (sense) or TRAIL-R DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of TRAIL-R DNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen (*Cancer Res.* 48:2659, 1988) and van der Krol et al. (*BioTechniques* 6:958, 1988).

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes. premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of TRAIL-R proteins. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable in vivo (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be

attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, an antisense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either in vivo or ex vivo. Suitable retroviral vectors include, but are not limited to, those derived from the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see WO 90/13641).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The following examples are provided to further illustrate particular embodiments of the invention, and are not to be construed as limiting the scope of the present invention.

EXAMPLE 1

Purification of TRAIL-R Protein

A human TRAIL receptor (TRAIL-R) protein was prepared by the following procedure. Trail-R was isolated from the cell membranes of Jurkat cells, a human acute T leukemia cell line. Jurkat cells were chosen because a specific band can be affinity precipitated from surface-biotinylated Jurkat cells, using Flag®-TRAIL covalently coupled to affi-gel beads (Biorad Laboratories, Richmond, Calif.). The precipitated band has a molecular weight of about 52 kD. A minor specific band of about 42 kD also was present, possibly accounting for a proteolytic breakdown product or a less glycosylated form of TRAIL-R.

Approximately 50 billion Jurkat cells were harvested by centrifugation (80 ml of cell pellet), washed once with PBS, then shock frozen on liquid nitrogen. Plasma membranes were isolated according to method number three described in Maeda et al., *Biochim. et Biophys. Acta,* 731:115, 1983; hereby incorporated by reference) with five modifications:

1. The following protease inhibitors were included in all solutions at the indicated concentrations: Aprotinin, 150 nM; EDTA, 5 mM; Leupeptin, 1 μM; pA-PMSF, 20 μM; Pefabloc, 500 μM; Pepstatin A, 1 μM; PMSF, 500 μM.
2. Dithiothreitol was not used.
3. DNAase was not used in the homogenization solution.
4. 1.25 ml of homogenization buffer was used per ml of cell pellet.
5. The homogenization was accomplished by five passages through a ground glass dounce homogenizer.

After isolation of the cell membranes, proteins were solubilized by resuspending the isolated membranes in 50 ml PBS containing 1% octylglucoside and all of the above mentioned protease inhibitors at the above indicated concentrations. The resulting solution was then repeatedly vortexed during a thirty-minute incubation at 4° C. The solution was then centrifuged at 20,000 rpm in an SW28 rotor in an LE-80 Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 4° C. for 30 minutes to obtain the supernatant (the membrane extract).

Chromatography

The first step of purification of TRAIL-R out of the membrane extract prepared above was affinity chromatography. The membrane extract was first applied to an anti-Flag®M2 affi-gel column (10 mg of monoclonal antibody M2 coupled to 2 ml of Affi-gel beads) to adsorb any nonspecifically binding material. The flow-through was then applied to a Flag®-TRAIL affi-gel column (10 mg of recombinant protein coupled to 1 ml of affi-gel beads).

The Affi-gel support is an N-hydroxysuccinimide ester of a derivatized, crosslinked agarose gel bead (available from Biorad Laboratories, Richmond, Calif.). As discussed above, the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, provides an epitope reversibly bound by specific monoclonal antibodies, enabling rapid assay and facile purification of expressed recombinant protein. M2 is a monoclonal antibody that binds Flag®. Monoclonal antibodies that bind the Flag® peptide, as well as other reagents for preparing and using Flag® fusion proteins, are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Conn. Preparation of Flag®-TRAIL fusion proteins (comprising Flag® fused to a soluble TRAIL polypeptide) is further described in PCT application WO 97/01633, hereby incorporated by reference.

The column was washed with 25 ml of each of the following buffers, in the order indicated:

1. PBS containing 1% octylglucoside
2. PBS
3. PBS containing an additional 200 mM NaCl
4. PBS

The bound material was eluted with 50 mM Na Citrate (pH 3) in 1 ml fractions and immediately neutralized with 300 μl of 1 M Tris-HCl (pH 8.5) per fraction. The TRAIL-binding activity of each fraction was determined by a TRAIL-R-specific ELISA as described below. Fractions with high TRAIL-binding activity were pooled, brought to 0.1% Trifluoroacetic acid (TFA), and subsequently chromatographed on a capillary reversed-phase HPLC column [500 μm internal diameter×25 cm fused silicone capillary column packed with 5 μm Vydac C_4 material (Vydac, Hesperia, Calif.)] using a linear gradient (2% per minute) from 0% to 100% acetonitrile in water containing 0.1% TFA. Fractions containing high TRAIL-binding activity are then determined as above, pooled, and, if desired, lyophilized.

TRAIL-R-specific ELISA:

Serial dilutions of TRAIL-R-containing samples (in 50 mM $NaHCO_3$, brought to pH 9 with NaOH) were coated onto Linbro/Titertek 96 well flat bottom E.I.A. microtitration plates (ICN Biomedicals Inc., Aurora, Ohio) at 100 μl/well. After incubation at 4° C. for 16 hours, the wells were washed six times with 200 μl PBS containing 0.05% Tween-

20 (PBS-Tween). The wells were then incubated with Flag®-TRAIL at 1 μg/ml in PBS-Tween with 5% fetal calf serum (FCS) for 90 minutes (100 μl per well), followed by washing as above. Next, each well was incubated with the anti-Flag® monoclonal antibody M2 at 1 μg/ml in PBS-Tween containing 5% FCS for 90 minutes (100 μl per well), followed by washing as above. Subsequently, wells were incubated with a polyclonal goat anti-mIgG1-specific horseradish peroxidase-conjugated antibody (a 1:5000 dilution of the commercial stock in PBS-Tween containing 5% FCS) for 90 minutes (100 μl per well). The HRP-conjugated antibody was obtained from Southern Biotechnology Associates, Inc., Birmingham, Ala. Wells then were washed six times, as above.

For development of the ELISA, a substrate mix [100 μl per well of a 1:1 premix of the TMB Peroxidase Substrate and Peroxidase Solution B (Kirkegaard Perry Laboratories, Gaithersburg, Md.)] was added to the wells. After sufficient color reaction, the enzymatic reaction was terminated by addition of 2 N H_2SO_4 (50 μl per well). Color intensity (indicating TRAIL-binding activity) was determined by measuring extinction at 450 nm on a V Max plate reader (Molecular Devices, Sunnyvale, Calif.).

EXAMPLE 2

Amino Acid Sequence

(a) TRAIL-R purified from Jurkat cells

TRAIL-R protein isolated from Jurkat cells was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on one of the peptide fragments produced by the tryptic digest. The fragment was found to contain the following sequence, which corresponds to amino acids 327 to 333 of the sequence presented in SEQ ID NO:2: VPANEGD.

(b) TRAIL-R purified from PS-1 cells

TRAIL-R protein was also isolated from PS-1 cells. PS-1 is a human B cell line that spontaneously arose after lethal irradiation of human peripheral blood lymphocytes (PBLs). The TRAIL-R protein was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on peptide fragments that resulted from the tryptic digest. One of the fragments was found to contain the following sequence, which, like the fragment presented in (a), corresponds to amino acids 327 to 333 of the sequence presented in SEQ ID NO:2: VPANEGD.

EXAMPLE 3

DNA and Amino Acid Sequences

The amino acid sequence of additional tryptic digest peptide fragments of TRAIL-R was determined. Degenerate oligonucleotides, based upon the amino acid sequence of two of the peptides, were prepared. A TRAIL-R DNA fragment was isolated and amplified by polymerase chain reaction (PCR), using the degenerate oligonucleotides as 5' and 3' primers. The PCR was conducted according to conventional procedures, using cDNA derived from the PS-1 cell line described in example 2 as the template. The nucleotide sequence of the isolated TRAIL-R DNA fragment (excluding portions corresponding to part of the primers), and the amino acid sequence encoded thereby, are presented in FIG. **1** (SEQ ID NOS:3 and 4). The sequence of the entire TRAIL-R DNA fragment isolated by PCR corresponds to nucleotides 988 to 1164 of SEQ ID NO:1, which encode amino acids 330 to 388 of SEQ ID NO:2.

The amino acid sequence in SEQ ID NO:4 bears significant homology to the so-called death domains found in certain other receptors. The cytoplasmic region of Fas and TNF receptor type I each contain a death domain, which is associated with transduction of an apoptotic signal (Tartaglia et al. *Cell* 74:845, 1993; Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993). Thus, the sequence presented in SEQ ID NO:4 is believed to be found within the cytoplasmic domain of TRAIL-R.

A probe derived from the fragment isolated above was used to screen a cDNA library (human foreskin fibroblast-derived cDNA in λgt10 vector), and a human TRAIL-R cDNA was isolated. The nucleotide sequence of the coding region of this cDNA is presented in SEQ ID NO:1, and the amino acid sequence encoded thereby is shown in SEQ ID NO:2.

EXAMPLE 4

Monoclonal Antibodies That Bind TRAIL-R

This example illustrates a method for preparing monoclonal antibodies that bind TRAIL-R. Suitable immunogens that may be employed in generating such antibodies include, but are not limited to, purified TRAIL-R protein or an immunogenic fragment thereof such as the extracellular domain, or fusion proteins containing TRAIL-R (e.g., a soluble TRAIL-R/Fc fusion protein).

Purified TRAIL-R can be used to generate monoclonal antibodies immunoreactive therewith, using conventional techniques such as those described in U.S. Pat. No. 4,411,993. Briefly, mice are immunized with TRAIL-R immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10–100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional TRAIL-R emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for TRAIL-R antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of TRAIL binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL-R in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3×63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL-R by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Pat. No. 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL-R monoclonal antibodies. Alternatively, hybridoma cells can be grown in vitro in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to Protein A or Protein G can also be used, as can affinity chromatography based upon binding to TRAIL-R.

EXAMPLE 5

Northern Blot Analysis

The tissue distribution of TRAIL-R mRNA was investigated by Northern blot analysis, as follows. An aliquot of a radiolabeled probe (the same radiolabeled probe used to screen the cDNA library in example 3) was added to two different human multiple tissue Northern blots (Clontech, Palo Alto, Calif.; Biochain, Palo Alto, Calif.). Hybridization was conducted overnight at 63° C. in 50% formamide as previously described (March et al., *Nature* 315:641–647, 1985). The blots then were washed with 2×SSC, 0.1% SDS at 68° C. for 30 minutes. A glycerol-aldehyde-phosphate dehydrogenase (GAPDH) specific probe was used to standardize for RNA loadings.

A single transcript of 4.4 kilobases (kb) was present in all tissues examined, including spleen, thymus, peripheral blood lymphocytes (PBLs), prostate, testis, ovary, uterus, placenta, and multiple tissues along the gastro-intestinal tract (including esophagus, stomach, duodenum, jejunum/ileum, colon, rectum, and small intestine). The cells and tissues with the highest levels of TRAIL-R mRNA are PBLs, spleen, and ovary, as shown by comparison to control hybridizations with a GAPDH-specific probe.

EXAMPLE 6

Binding Assay

Full length human TRAIL-R was expressed and tested for the ability to bind TRAIL. The binding assay was conducted as follows.

A fusion protein comprising a leucine zipper peptide fused to the N-terminus of a soluble TRAIL polypeptide (LZ-TRAIL) was employed in the assay. An expression construct was prepared, essentially as described for preparation of the Flag®-TRAIL expression construct in Wiley et al. (*Immunity,* 3:673–682, 1995; hereby incorporated by reference), except that DNA encoding the Flag® peptide was replaced with a sequence encoding a modified leucine zipper that allows for trimerization. The construct, in expression vector pDC409, encoded a leader sequence derived from human cytomegalovirus, followed by the leucine zipper moiety fused to the N-terminus of a soluble TRAIL polypeptide. The TRAIL polypeptide comprised amino acids 95–281 of human TRAIL (a fragment of the extracellular domain), as described in Wiley et al. (supra). The LZ-TRAIL was expressed in CHO cells, and purified from the culture supernatant.

The expression vector designated pDC409 is a mammalian expression vector derived from the pDC406 vector described in McMahan et al. (*EMBO J.* 10:2821–2832, 1991; hereby incorporated by reference). Features added to pDC409 (compared to pDC406) include additional unique restriction sites in the multiple cloning site (mcs); three stop codons (one in each reading frame) positioned downstream of the mcs; and a T7 polymerase promoter, downstream of the mcs, that faciliates sequencing of DNA inserted into the mcs.

For expression of full length human TRAIL-R protein, the entire coding region (i.e., the DNA sequence presented in SEQ ID NO:1) was amplified by polymerase chain reaction (PCR). The template employed in the PCR was the cDNA clone isolated from a human foreskin fibroblast cDNA library, as described in example 3. The isolated and amplified DNA was inserted into the expression vector pDC409, to yield a construct designated pDC409-TRAIL-R.

CrmA protein was employed to inhibit apoptosis of host cells expressing recombinant TRAIL-R, as discussed above and in example 8. An expression vector designated pDC409-CrmA contained DNA encoding poxvirus CrmA in pDC409. The 38-kilodalton cowpox-derived protein that was subsequently designated CrmA is described in Pickup et al. (*Proc. Natl. Acad. Sci. USA* 83:7698–7702, 1986; hereby incorporated by reference).

CV-1/EBNA cells were co-transfected with pDC409-TRAIL-R together with pDC409-CrmA, or with pDC409-CrmA alone. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. 48 hours after transfection, cells were detached non-enzymatically and incubated with LZ-TRAIL (5 μg/ml), a biotinylated anti-LZ monoclonal antibody (5 μg/ml), and phycoerythrin-conjugated streptavidin (1:400), before analysis by fluorescence-activated cell scanning (FACS). The cytometric analysis was conducted on a FACscan (Beckton Dickinson, San Jose, Calif.).

The CV-1/EBNA cells co-transfected with vectors encoding TRAIL-R and CrmA showed significantly enhanced binding of LZ-TRAIL, compared to the cells transfected with the CrmA-encoding vector alone.

EXAMPLE 7

TRAIL-R Blocks TRAIL-Induced Apoptosis of Target Cells

TRAIL-R was tested for the ability to block TRAIL-induced apoptosis of Jurkat cells. The TRAIL-R employed in the assay was in the form of a fusion protein designated sTRAIL-R/Fc, which comprised the extracellular domain of human TRAIL-R, fused to the N-terminus of an Fc polypeptide derived from human IgG1.

CV1-EBNA cells were transfected with a recombinant expression vector comprising a gene fusion encoding the sTRAIL-R/Fc protein, in the pDC409 vector described in example 6, and cultured to allow expression of the fusion protein. The sTRAIL-R/Fc fusion protein was recovered from the culture supernatant. Procedures for fusing DNA encoding an IgG1 Fc polypeptide to DNA encoding a heterologous protein are described in Smith et al., (*Cell* 73:1349–1360, 1993); analogous procedures were employed herein.

A fusion protein designated TNF-R2-Fc, employed as a control, comprised the extracellular domain of the TNF receptor protein known as p75 or p80 TNF-R (Smith et al., *Science* 248:1019–1023, 1990; Smith et al. *Cell.*76:959–962, 1994), fused to an Fc polypeptide. A mouse monoclonal antibody that is a blocking antibody directed against human TRAIL, was employed in the assay as well.

Jurkat cells were incubated with varying or constant concentrations of LZ-TRAIL (the LZ-TRAIL fusion protein described in example 6), in the absence or presence of varying concentrations of sTRAIL-R-Fc, TNF-R2-Fc, or the TRAIL-specific monoclonal antibody. Cell death was quantitated using an MTT cell viability assay (MTT is 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), as previously described (Mosmann, *J. Immunol. Methods* 65:55–63, 1983). The results are shown in FIG. **2**, which presents the percent cell death for Jurkat cells that were untreated (Δ) or were treated with varying (▲) or constant (○, ●, □, ■) concentrations of LZ-TRAIL (13 ng/ml) in the absence (●) or presence of varying concentrations of TRAIL-R2-Fc (■), TNF-R2-Fc (□), or the anti-TRAIL

antibody (○). Varying concentrations for all substances started at 10 μg/ml and were serially diluted.

The anti-TRAIL monoclonal antibody and sTRAIL-R/Fc each blocked TRAIL-induced apoptosis in a dose dependent fashion, whereas TNFR2-Fc did not. Thus, the extracellular domain of TRAIL-R is capable of binding to TRAIL and inhibiting TRAIL-mediated apoptosis of target cells.

EXAMPLE 8

TRAIL-R-induced Apoptosis is Blocked by Caspase Inhibitors and FADD-DN

CV-1/EBNA cells were transfected, by the DEAE-dextran method, with expression plasmids for TRAIL-R (pDC409-TRAIL-R), together with a threefold excess of empty expression vector (pDC409) in the presence or absence of z-VAD-fmk (10 μM; in the culture medium), or together with a threefold excess of expression vector pDC409-CrmA, pDC409-p35, or pDC409-FADD-DN. In addition, 400 ng/slide of an expression vector for the *E. coli* lacz gene was co-transfected together with all DNA mixes. The transfected cells were cultured in chambers mounted on slides.

The mammalian expression vector pDC409, and the pDC409-TRAIL-R vector encoding full length human TRAIL-R, are described in example 6. The tripeptide derivative zVAD-fmk (benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone) is available from Enzyme System Products, Dublin, Calif.

The 38-kilodalton cowpox-derived protein that was subsequently designated CrmA is described in Pickup et al. (*Proc. Natl. Acad. Sci. USA* 83:7698–7702, 1986; hereby incorporated by reference). Sequence information for CrmA is presented in FIG. 4 of Pickup et al., supra.

A 35-kilodalton protein encoded by *Autographa californica* nuclear polyhedrosis virus, a baculovirus, is described in Friesen and Miller (*J Virol.* 61:2264–2272, 1987; hereby incorporated by reference). Sequence information for this protein, designated baculovirus p35 herein, is presented in FIG. 5 of Friesen and Miller, supra.

FADD (also designated MORT1) is described in Boldin et al. (*J. Biol. Chem.* 270:7795–7798, 1995; hereby incorporated by reference). The protein referred to as FADD-DN (FADD dominant negative) is a C-terminal fragment of FADD that includes the death domain. DNA encoding FADD-DN, fused to an N-terminal Flag® epitope tag (described above), was inserted into the pDC409 expression vector described in example 6, to form pDC409-FADD-DN. The FADD-DN polypeptide corresponds to amino acids 117 through 245 of the MORT1 amino acid sequence presented in Boldin et al., supra.

48 hours after transfection, cells were washed with PBS, fixed with glutaraldehyde and incubated with X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside). Cells expressing β-galactosidase stain blue. A decrease in the percentage of stained cells indicates loss of β-galactosidase expression, and correlates with death of cells that express the protein(s) co-transfected with the lacz gene.

The results are presented in FIG. **3**, wherein the values plotted represent the mean and standard deviation of at least three separate experiments. Poxvirus CrmA, baculovirus p35, FADD-DN, and z-VAD-fmk each effectively reduced death of transfected cells expressing TRAIL-R.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1323 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: huTrail-R

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1323

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG GAA CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG AAA       48
Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg Lys
  1               5                  10                  15

AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC AGG CCT GGG CCC       96
Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
             20                  25                  30
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CGG GTC CCC AAG ACC CTT GTG CTC GTT GTC GCC GCG GTC CTG CTG TTG      144
Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
          35                  40                  45

GTC TCA GCT GAG TCT GCT CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG      192
Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
     50                  55                  60

CAG AGA GCG GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG      240
Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
 65                  70                  75                  80

TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT TGC ATC TCC      288
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
                 85                  90                  95

TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC TGG AAT GAC CTC CTT TTC      336
Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
            100                 105                 110

TGC TTG CGC TGC ACC AGG TGT GAT TCA GGT GAA GTG GAG CTA AGT CCG      384
Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
        115                 120                 125

TGC ACC ACG ACC AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC      432
Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
    130                 135                 140

CGG GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA GGG TGT      480
Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145                 150                 155                 160

CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA CCC TGG AGT GAC ATC      528
Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
                165                 170                 175

GAA TGT GTC CAC AAA GAA TCA GGT ACA AAG CAC AGT GGG GAA GCC CCA      576
Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
            180                 185                 190

GCT GTG GAG GAG ACG GTG ACC TCC AGC CCA GGG ACT CCT GCC TCT CCC      624
Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
        195                 200                 205

TGT TCT CTC TCA GGC ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC      672
Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
    210                 215                 220

TTG ATT GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA GTC      720
Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
225                 230                 235                 240

CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT GGG GAC CCT GAG      768
Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
                245                 250                 255

CGT GTG GAC AGA AGC TCA CAA CGA CCT GGG GCT GAG GAC AAT GTC CTC      816
Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
            260                 265                 270

AAT GAG ATC GTG AGT ATC TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA      864
Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
        275                 280                 285

ATG GAA GTC CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC      912
Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
    290                 295                 300

CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT GAA AGG TCT      960
Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala Glu Arg Ser
305                 310                 315                 320

CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT GAA GGT GAT CCC ACT GAG     1008
Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Glu Gly Asp Pro Thr Glu
                325                 330                 335

ACT CTG AGA CAG TGC TTC GAT GAC TTT GCA GAC TTG GTG CCC TTT GAC     1056
Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe Asp
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            340                 345                 350

TCC TGG GAG CCG CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA     1104
Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile
        355                 360                 365

AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG TAC ACG     1152
Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Tyr Thr
    370                 375                 380

ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA GAT GCC TCT GTC CAC     1200
Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala Ser Val His
385                 390                 395                 400

ACC CTG CTG GAT GCC TTG GAG ACG CTG GGA GAG AGA CTT GCC AAG CAG     1248
Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln
                405                 410                 415

AAG ATT GAG GAC CAC TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA     1296
Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu
            420                 425                 430

GGT AAT GCA GAC TCT GCC ATG TCC TAA                                 1323
Gly Asn Ala Asp Ser Ala Met Ser  *
        435                 440


(2) INFORMATION FOR SEQ ID NO: 2:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 440 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg Lys
  1               5                  10                  15

Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
             20                  25                  30

Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
         35                  40                  45

Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
     50                  55                  60

Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
 65                  70                  75                  80

Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
                 85                  90                  95

Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
            100                 105                 110

Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
        115                 120                 125

Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
    130                 135                 140

Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145                 150                 155                 160

Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
                165                 170                 175

Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
            180                 185                 190

Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
        195                 200                 205

Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
    210                 215                 220
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Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
225                 230                 235                 240

Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
                245                 250                 255

Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
            260                 265                 270

Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
        275                 280                 285

Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
    290                 295                 300

Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala Glu Arg Ser
305                 310                 315                 320

Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Glu Gly Asp Pro Thr Glu
                325                 330                 335

Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe Asp
            340                 345                 350

Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile
        355                 360                 365

Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Tyr Thr
    370                 375                 380

Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala Ser Val His
385                 390                 395                 400

Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln
                405                 410                 415

Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu
            420                 425                 430

Gly Asn Ala Asp Ser Ala Met Ser
        435                 440
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(B) CLONE: huTrail-R frag

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
CT GAG ACT CTG AGA CAG TGC TTC GAT GAC TTT GCA GAC TTG GTG CCC        47

   Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro
     1               5                  10                  15

TTT GAC TCC TGG GAG CCG CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT       95
Phe Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn
                 20                  25                  30

GAG ATA AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG      143
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-continued

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Glu Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu
            35                  40                  45

TNC ACN ATG CTG AT                                              157
Xaa Thr Met Leu
         50


(2) INFORMATION FOR SEQ ID NO: 4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 51 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe
  1               5                  10                  15

Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu
             20                  25                  30

Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Xaa
         35                  40                  45

Thr Met Leu
     50


(2) INFORMATION FOR SEQ ID NO: 5:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 8 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

   (vii) IMMEDIATE SOURCE:
          (B) CLONE: FLAG peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asp Tyr Lys Asp Asp Asp Asp Lys
  1               5
```

What is claimed is:

1. A purified TRAIL-R polypeptide selected from the group consisting of:

a) the TRAIL-R polypeptide of SEQ ID NO:2; and

b) a fragment of the polypeptide of (a), wherein said fragment binds TRAIL.

2. A TRAIL-R polypeptide of claim **1**, wherein said polypeptide comprises amino acids x to 440 of SEQ ID NO:2, wherein x represents an integer from 51 through 59.

3. A TRAIL-R polypeptide of claim **2**, wherein said polypeptide comprises amino acids 54 to 440 of SEQ ID NO:2.

4. A TRAIL-R polypeptide of claim **1**, wherein said fragment is a soluble TRAIL-R fragment.

5. A purified TRAIL-R polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence presented in SEQ ID NO:2 wherein said polypeptide binds TRAIL.

6. A TRAIL-R polypeptide of claim **5**, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence presented in SEQ ID NO:2.

7. A TRAIL-R polypeptide of claim **6**, wherein said polypeptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence presented in SEQ ID NO:2.

8. An oligomer comprising from two to four TRAIL-R polypeptides of claim **5**.

9. An oligomer comprising from two to four TRAIL-R polypeptides of claim **8**.

10. A composition comprising a TRAIL-R polypeptide of claim **1**, and a physiologically acceptable diluent, excipient, or carrier.

11. A composition comprising a TRAIL-R polypeptide of claim **4**, and a physiologically acceptable diluent, excipient, or carrier.

12. A purified polypeptide encoded by a DNA comprising the nucleotide sequence of SEQ ID NO:1.

13. A purified polypeptide comprising amino acids x to 210 of SEQ ID NO:2, wherein x represents an integer from 51 to 59, wherein said polypeptide binds TRAIL.

14. A polypeptide of claim **13**, wherein said polypeptide comprises amino acids 54 to 210 of SEQ ID NO:2.

15. A polypeptide of claim wherein said polypeptide comprises amino acids 52 to 210 of SEQ ID NO:2.

16. A polypeptide of claim **13**, wherein said polypeptide is a soluble TRAIL-R polypeptide.

17. A polypeptide of claim **1**, wherein said fragment comprises amino acids 94 to 178 SEQ ID NO:2.

18. A purified polypeptide comprising amino acids 94 to 178 of SEQ ID NO:2, wherein said polypeptide binds TRAIL.

19. A purified soluble TRAIL-R polypeptide comprising an amino acid sequence that is at least 80% identical to the sequence of amino acids 54 to 210 of SEQ ID NO:2, wherein said polypeptide binds TRAIL.

20. A polypeptide of claim **19**, wherein the polypeptide comprises an amino acid sequence that is at least 90% identical to the sequence of amino acids 54 to 210 of SEQ ID NO:2.

21. A polypeptide of claim **19**, wherein the polypeptide comprises an amino acid sequence that differs from the sequence of amino acids 54 to 210 of SEQ ID NO:2 by from one to ten amino acid deletions, insertions, or substitutions.

22. An oligomer comprising at least two polypeptides of claim **13**.

23. An oligomer comprising at least two polypeptides of claim **14**.

24. An oligomer comprising at least two polypeptides of claim **15**.

25. An oligomer comprising at least two polypeptides of claim **16**.

26. An oligomer comprising at least two polypeptides of claim **17**.

27. An oligomer comprising at least two polypeptides of claim **18**.

28. An oligomer comprising at least two polypeptides of claim **19**.

29. An oligomer comprising at least two polypeptides of claim **20**.

30. An oligomer comprising at least two polypeptides of claim **21**.

31. An oligomer of claim **22**, wherein the oligomer comprises two or three of said polypeptides.

32. An oligomer of claim **23**, wherein the oligomer comprises two or three of said polypeptides.

33. An oligomer of claim **24**, wherein the oligomer comprises two or three of said polypeptides.

34. An oligomer of claim **27**, wherein the oligomer comprises two or three of said polypeptides.

35. An oligomer of claim **31**, wherein the oligomer comprises two of said polypeptides.

36. A composition comprising a polypeptide of claim **13**, and a physiologically acceptable diluent, excipient, or carrier.

37. A composition comprising a polypeptide of claim **18**, and a physiologically acceptable diluent, excipient, or carrier.

38. A composition comprising an oligomer of claim **9**, and a physiologically acceptable diluent, excipient, or carrier.

39. A composition comprising an oligomer of claim **22**, and a physiologically acceptable diluent, excipient, or carrier.

40. A composition comprising an oligomer of claim **27**, and a physiologically acceptable diluent, excipient, or carrier.

41. A composition comprising an oligomer of claim **31**, and a physiologically acceptable diluent, excipient, or carrier.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,642,358 B1
DATED : November 4, 2003
INVENTOR(S) : Charles Rauch and Henning Walczak

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 12,
Line 32, "interleukin4" should read -- interleukin-4 --.

Column 16,
Lines 61 and 66, "TRIAL" should read -- TRAIL --.

Column 17,
Lines 2, 6 and 7, "TRIAL" should read -- TRAIL --.
Line 6, "defection" should read -- defective --.

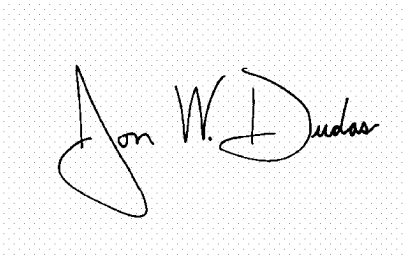
Column 18,
Line 21, "325" should read -- 3245 --.

Column 38,
Line 55, "5" should read -- 1 --.
Line 57, "8" should read -- 4 --.

Column 39,
Line 3, should read -- A polypeptide of claim 13 --.

Signed and Sealed this

Thirty-first Day of January, 2006



JON W. DUDAS
Director of the United States Patent and Trademark Office

JS 44 (Rev. 3/99)

CIVIL COVER SHEET

The JS-44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. (SEE INSTRUCTIONS ON THE REVERSE OF THE FORM.)

I. (a) PLAINTIFFS

HUMAN GENOME SCIENCES, INC.

(b) County of Residence of First Listed Plaintiff *
(EXCEPT IN U.S. PLAINTIFF CASES)

* Plaintiff is a Delaware corporation

DEFENDANTS

AMGEN INC. and IMMUNEX CORP.

County of Residence of First Listed _____
(IN U.S. PLAINTIFF CASES ONLY)
NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE LAND INVOLVED.

(c) Attorney's (Firm Name, Address, and Telephone Number)
Steven J. Balick
Ashby & Geddes
500 Delaware Avenue, 8th FL., P.O. Box 1150
Wilmington, DE 19899 (302) 654-1888

Attorneys (If Known)

Unknown

II. BASIS OF JURISDICTION (Place an "X" in One Box Only)

- ☐ 1 U.S. Government Plaintiff
- ☒ 3 Federal Question (U.S. Government Not a Party)
- ☐ 2 U.S. Government Defendant
- ☐ 4 Diversity (Indicate Citizenship of Parties in Item III)

III. CITIZENSHIP OF PRINCIPAL PARTIES (Place an "X" in One Box for Plaintiff and One Box for Defendant)
(For Diversity Cases Only)

		DEF			DEF
Citizen of This State	☐ 1	☐ 1	Incorporated *or* Principal Place of Business In This State	☐ 4	☐ 4
Citizen of Another State	☐ 2	☐ 2	Incorporated *and* Principal Place of Business In Another State	☐ 5	☐ 5
Citizen or Subject of a Foreign Country	☐ 3	☐ 3	Foreign Nation	☐ 6	☐ 6

IV. NATURE OF SUIT (Place an "X" in One Box Only)

CONTRACT
- ☐ 110 Insurance
- ☐ 120 Marine
- ☐ 130 Miller Act
- ☐ 140 Negotiable Instrument
- ☐ 150 Recovery of Overpayment & Enforcement of Judgment
- ☐ 151 Medicare Act
- ☐ 152 Recovery of Defaulted Student Loans (Excl. Veterans)
- ☐ 153 Recovery of Overpayment of Veteran's Benefits
- ☐ 160 Stockholders' Suits
- ☐ 190 Other Contract
- ☐ 195 Contract Product Liability

REAL PROPERTY
- ☐ 210 Land Condemnation
- ☐ 220 Foreclosure
- ☐ 230 Rent Lease & Ejectment
- ☐ 240 Torts to Land
- ☐ 245 Tort Product Liability
- ☐ 290 All Other Real Property

TORTS

PERSONAL INJURY
- ☐ 310 Airplane
- ☐ 315 Airplane Product Liability
- ☐ 320 Assault, Libel & Slander
- ☐ 330 Federal Employers' Liability
- ☐ 340 Marine
- ☐ 345 Marine Product Liability
- ☐ 350 Motor Vehicle
- ☐ 355 Motor Vehicle Product Liability
- ☐ 360 Other Personal Injury

PERSONAL INJURY
- ☐ 362 Personal Injury—Med. Malpractice
- ☐ 365 Personal Injury—Product Liability
- ☐ 368 Asbestos Personal Injury Product Liability

PERSONAL PROPERTY
- ☐ 370 Other Fraud
- ☐ 371 Truth in Lending
- ☐ 380 Other Personal Property Damage
- ☐ 385 Property Damage Product Liability

CIVIL RIGHTS
- ☐ 441 Voting
- ☐ 442 Employment
- ☐ 443 Housing/ Accommodations
- ☐ 444 Welfare
- ☐ 440 Other Civil Rights

PRISONER PETITIONS
- ☐ 510 Motions to Vacate Sentence
- Habeas Corpus:
- ☐ 530 General
- ☐ 535 Death Penalty
- ☐ 540 Mandamus & Other
- ☐ 550 Civil Rights
- ☐ 555 Prison Condition

FORFEITURE/PENALTY
- ☐ 610 Agriculture
- ☐ 620 Other Food & Drug
- ☐ 625 Drug Related Seizure of Property 21 USC
- ☐ 630 Liquor Laws
- ☐ 640 R.R. & Truck
- ☐ 650 Airline Regs.
- ☐ 660 Occupational Safety/Health
- ☐ 690 Other

LABOR
- ☐ 710 Fair Labor Standards Act
- ☐ 720 Labor/Mgmt. Relations
- ☐ 730 Labor/Mgmt.Reporting & Disclosure Act
- ☐ 740 Railway Labor Act
- ☐ 790 Other Labor Litigation
- ☐ 791 Empl. Ret. Inc. Security Act

BANKRUPTCY
- ☐ 422 Appeal 28 USC 158
- ☐ 423 Withdrawal 28 USC 157

PROPERTY RIGHTS
- ☐ 820 Copyrights
- ☒ 830 Patent
- ☐ 840 Trademark

SOCIAL SECURITY
- ☐ 861 HIA (1395ff)
- ☐ 862 Black Lung (923)
- ☐ 863 DIWC/DIWW (405(g))
- ☐ 864 SSID Title XVI
- ☐ 865 RSI (405(g))

FEDERAL TAX SUITS
- ☐ 870 Taxes (U.S. Plaintiff or Defendant)
- ☐ 871 IRS—Third Party 26 USC 7609

OTHER STATUTES
- ☐ 400 State Reapportionment
- ☐ 410 Antitrust
- ☐ 430 Banks and Banking
- ☐ 450 Commerce/ICC Rates/etc.
- ☐ 460 Deportation
- ☐ 470 Racketeer Influenced and Corrupt Organizations
- ☐ 810 Selective Service
- ☐ 850 Securities/Commodities/ Exchange
- ☐ 875 Customer Challenge 12 USC 3410
- ☐ 891 Agricultural Acts
- ☐ 892 Economic Stabilization Act
- ☐ 893 Environmental Matters
- ☐ 894 Energy Allocation Act
- ☐ 895 Freedom of Information Act
- ☐ 900Appeal of Fee Determination Under Equal Access to Justice
- ☐ 950 Constitutionality of State Statutes
- ☐ 890 Other Statutory Actions

V. ORIGIN (PLACE AN "X" IN ONE BOX ONLY)

☒ 1 Original Proceeding ☐ 2 Removed from State Court ☐ 3 Remanded from Appellate Court ☐ 4 Reinstated or Reopened ☐ 5 Transferred from another district (specify) ☐ 6 Multidistrict Litigation ☐ 7 Appeal to District Judge from Magistrate Judgment

VI. CAUSE OF ACTION (Cite the U.S. Civil Statute under which you are filing and write brief statement of cause. Do not cite jurisdictional statutes unless diversity.)

Action under 35 U.S.C. § 146 to review a judgment entered by the Board of Patent Appeals and Interferences.

VII. REQUESTED IN COMPLAINT: ☐ CHECK IF THIS IS A CLASS ACTION UNDER F.R.C.P. 23 **DEMAND $** CHECK YES only if demanded in complaint: **JURY DEMAND:** ☐ Yes ☒ No

VIII. RELATED CASE(S) IF ANY (See instructions): JUDGE _____ DOCKET NUMBER _____

DATE August 30, 2007

SIGNATURE OF ATTORNEY OF RECORD Steven J. Balick

FOR OFFICE USE ONLY

RECEIPT # _____ AMOUNT _____ APPLYING IFP _____ JUDGE _____ MAG. JUDGE _____

JS 44 Reverse (Rev. 12/96)

INSTRUCTIONS FOR ATTORNEYS COMPLETING CIVIL COVER SHEET FORM JS-44

Authority For Civil Cover Sheet

The JS-44 civil cover sheet and the information contained herein neither replaces nor supplements the filings and service of pleading or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. Consequently, a civil cover sheet is submitted to the Clerk of Court for each civil complaint filed. The attorney filing a case should complete the form as follows:

I. **(a) Plaintiffs-Defendants.** Enter names (last, first, middle initial) of plaintiff and defendant. If the plaintiff or defendant is a government agency, use only the full name or standard abbreviations. If the plaintiff or defendant is an official within a government agency, identify first the agency and then the official, giving both name and title.

(b.) County of Residence. For each civil case filed, except U.S. plaintiff cases, enter the name of the county where the first listed plaintiff resides at the time of filing. In U.S. plaintiff cases, enter the name of the county in which the first listed defendant resides at the time of filing. (NOTE: In land condemnation cases, the county of residence of the "defendant" is the location of the tract of land involved.)

(c) Attorneys. Enter the firm name, address, telephone number, and attorney of record. If there are several attorneys, list them on an attachment, noting in this section "(see attachment)".

II. **Jurisdiction**. The basis of jurisdiction is set forth under Rule 8(a), F.R.C.P., which requires that jurisdictions be shown in pleadings. Place an "X" in one of the boxes. If there is more than one basis of jurisdiction, precedence is given in the order shown below.

United States plaintiff. (1) Jurisdiction based on 28 U.S.C. 1345 and 1348. Suits by agencies and officers of the United States, are included here.

United States defendant. (2) When the plaintiff is suing the United States, its officers or agencies, place an "X" in this box.

Federal question. (3) This refers to suits under 28 U.S.C. 1331, where jurisdiction arises under the Constitution of the United States, an amendment to the Constitution, an act of Congress or a treaty of the United States. In cases where the U.S. is a party, the U.S. plaintiff or defendant code takes precedence, and box 1 or 2 should be marked.

Diversity of citizenship. (4) This refers to suits under 28 U.S.C. 1332, where parties are citizens of different states. When Box 4 is checked, the citizenship of the different parties must be checked. (See Section III below; federal question actions take precedence over diversity cases.)

III. **Residence (citizenship) of Principal Parties.** This section of the JS-44 is to be completed if diversity of citizenship was indicated above. Mark this section for each principal party.

IV. **Nature of Suit.** Place an "X" in the appropriate box. If the nature of suit cannot be determined, be sure the cause of action, in Section IV below, is sufficient to enable the deputy clerk or the statistical clerks in the Administrative Office to determine the nature of suit. If the cause fits more than one nature of suit, select the most definitive.

V. **Origin.** Place an "X" in one of the seven boxes.

Original Proceedings. (1) Cases which originate in the United States district courts.

Removed from State Court. (2) Proceedings initiated in state courts may be removed to the district courts under Title 28 U.S.C., Section 1441. When the petition for removal is granted, check this box.

Remanded from Appellate Court. (3) Check this box for cases remanded to the district court for further action. Use the date of remand as the filing date.

Reinstated or Reopened. (4) Check this box for cases reinstated or reopened in the district court. Use the reopening date as the filing date.

Transferred from Another District. (5) For cases transferred under Title 28 U.S.C. Section 1404(a) Do not use this for within district transfers or multidistrict litigation transfers.

Multidistrict Litigation. (6) Check this box when a multidistrict case is transferred into the district under authority of Title 28 U.S.C. Section 1407. When this box is checked, do not check (5) above.

Appeal to District Judge from Magistrate Judgment. (7) Check this box for an appeal from a magistrate judge's decision.

VI. **Cause of Action.** Report the civil statute directly related to the cause of action and give a brief description of the cause.

VII. **Requested in Complaint.** Class Action. Place an "X" in this box if you are filing a class action under Rule 23, F.R.Cv.P.

Demand. In this space enter the dollar amount (in thousands of dollars) being demanded or indicate other demand such as a preliminary injunction.

Jury Demand. Check the appropriate box to indicate whether or not a jury is being demanded.

VIII. **Related Cases.** This section of the JS-44 is used to reference related pending cases if any. If there are related pending cases, insert the docket numbers and the corresponding judge names for such cases.

Date and Attorney Signature. Date and sign the civil cover sheet.

AO FORM 85 RECEIPT (REV. 9/04)

United States District Court for the District of Delaware

Civil Action No. 07-526

ACKNOWLEDGMENT OF RECEIPT FOR AO FORM 85

NOTICE OF AVAILABILITY OF A UNITED STATES MAGISTRATE JUDGE TO EXERCISE JURISDICTION

I HEREBY ACKNOWLEDGE RECEIPT OF 3 COPIES OF AO FORM 85.

8/30/07
(Date forms issued)

Mike Bobish
(Signature of Party or their Representative)

Mike Bobish
(Printed name of Party or their Representative)

Note: Completed receipt will be filed in the Civil Action